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Early detection of the three major primary cancers in women by cell-free DNA methylation in liquid biopsies

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertação de Candidatura ao grau de **Mestre em Oncologia** – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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“Tão importante como fixar-se no essencial

é prescindir do secundário.”

Johann Josef Loschmidt

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RESUMO

Introdução: Os cancros da mama, colorretal e do pulmão são os três tipos de cancro mais comuns e mortais em mulheres de países desenvolvidos. O rastreio do cancro leva ao aumento do número de tumores detetados em estadios iniciais, estando, no entanto, associado a uma alta taxa de falsos positivos e ao tratamento de cancros possivelmente indolentes. A metilação aberrante do DNA é um fenómeno que ocorre cedo no desenvolvimento do cancro e que pode ser detetada em DNA tumoral circulante, constituindo um marcador promissor para a deteção precoce do cancro de uma forma não invasiva. Assim, este trabalho teve como principal objetivo desenvolver um teste sensível e específico baseado na metilação do DNA circulante extraído do plasma, para deteção simultânea dos cancros da mama, colorretal e pulmão.

Métodos: O DNA circulante foi extraído de amostras de plasma provenientes de pacientes com cancro da mama, colorretal, do pulmão e dadores saudáveis, sujeito a modificação bissulfito e a uma amplificação. Os níveis de metilação dos promotores dos genes *APC*, *FOXA1*, *MGMT*, *RARβ2*, *RASSF1A*, *SCGB3A1*, *SEPT9*, *SHOX2* e *SOX17* foram determinados por PCR específico de metilação em multiplex. Foram avaliadas as associações entre os níveis de metilação e variáveis clinicopatológicas. A sensibilidade e especificidade foram também calculadas de forma a avaliar a *performance* diagnóstica dos biomarcadores.

Resultados: O painel "PanCancer", constituído por *APC*, *FOXA1* e *RASSF1A*, detetou os três cancros mais frequentes nas mulheres com uma sensibilidade de 72% e uma especificidade de 74%, enquanto o painel "CancerType" (*SCGB3A1*, *SEPT9* e *SOX17*) indicou com alta especificidade (superior a 80%) a possível origem do tumor, embora com baixa sensibilidade.

Conclusões: A avaliação dos níveis de metilação em DNA circulante é uma estratégia promissora para o rastreio simultâneo dos cancros da mama, colorretal e do pulmão. Este teste tem o potencial para complementar os métodos atualmente usados, aperfeiçoar a triagem de indivíduos com suspeita de cancro, e aumentar a aderência aos programas de rastreio e o seu custo-efetividade.

ABSTRACT

Background: Breast (BrC), colorectal (CRC) and lung (LC) cancers are the three most common and deadly cancers in women in developed regions. Cancer screening entails increase in early stage disease detection but is variably hampered by high false-positive rates and overdiagnosis/overtreatment. Aberrant DNA methylation occurs early in cancer and may be detected in circulating cell-free DNA (ccfDNA), constituting a valuable cancer biomarker and enabling non-invasive testing for early cancer detection. Thus, we aimed to develop a sensitive and specific ccfDNA methylation-based test for simultaneous detection of BrC, CRC and LC.

Methods: CcfDNA from BrC, CRC and LC patients and healthy donors was extracted from plasma, sodium-bisulfite modified and whole-genome amplified. *APC, FOXA1, MGMT, RARβ2, RASSF1A, SCGB3A1*, *SEPT9*, *SHOX2* and *SOX17* promoter methylation levels were determined by multiplex quantitative methylation-specific PCR. Associations between methylation and standard clinicopathological parameters were assessed. Sensitivity and specificity were calculated to evaluate biomarkers' diagnostic performance.

Results: A "PanCancer" panel (*APC*, *FOXA1 and RASSF1A*) detected the three major cancers with 72% sensitivity and 74% specificity, whereas a "CancerType" panel (*SCGB3A1*, *SEPT9* and *SOX17*) complemented the former, to indicate the most likely cancer topography, with over 80% specificity, although with limited sensitivity.

Conclusions: Gene promoter methylation assessment in ccfDNA is promising for simultaneous screening of BrC, CRC and LC, complementing current screening modalities, perfecting the triage of cancer suspects, and increasing compliance and cost-effectiveness.

TABLE OF CONTENTS

FIGURES INDEX

TABLES INDEX

LIST OF ABBREVIATIONS

AC – Asymptomatic control

ACS - American Cancer Society

AJCC - American Joint Committee on Cancer

ALK - Anaplastic lymphoma kinase

APC – Adenomatosis polyposis coli

BrC - Breast cancer

BRCA - DNA repair associated

CcfDNA - Circulating cell-free tumor DNA

CEA - Carcinoembryogenic antigen

CIMP - CpG island methylator phenotype

CIN - Chromosomal instability

CpG - Cytosine-phosphate-guanine

CRC – Colorectal cancer

CT - Computed tomography

CTCs - Circulating tumor cells

DCIS - Ductal Carcinoma *In Situ*

DNMT - DNA methyltransferase

ER - Estrogen receptor

EGFR - Epidermal growth factor receptor

ERBB2 - Erb-B2 receptor tyrosine kinase 2

ESMO - European society for medical oncology

FAP – Familiar adenomatous polyposis

FISH - Fluorescence *in situ* hybridization

FIT - Feacal immunochemical test

FNA - Fine-needle aspiration

FOBT - Faecal occult blood testing

FOXA1 – Fork-head box A1

gFOBT - Guaiac-based faecal occult blood testing

LC – Lung cancer

LCIS – Lobular carcinoma *in situ*

LDCT - Low-dose computed tomography

HER2 - Human epidermal growth factor receptor 2

HNPCC – Hereditary nonpolyposis colorectal cancer

HT – Hormone therapy

IHC - Immunohistochemistry

MBP - Methyl-CpG-binding protein

MDA – Multiple displacement amplification

MGMT – 6-methylguanine DNA methyltransferase

MMR - Mismatch repair

MRI - Magnetic resonance imaging

MSI - Microsatellite instability

NELSON - Dutch-Belgian Lung Cancer Screening Trial

NNK - 4-(methylnitrosamino)-1-(13-pyridyl-1-butanone)

NLST - National Lung Screening Trial

NPV – Negative predictive value

NSCLC - Non-small cell lung cancer

NSAIDS - Nonsteroidal anti-inflammatory drugs

PD-L1 - Programmed death-ligand 1

PET - Positron emission tomography

PPV – Positive predictive value

PR – Progesterone receptor

qMSP – Quantitative methylation-specific PCR

RARβ2 – Retinoic acid receptor beta 2

RASSF1A – Ras association domain family 1 isoform A

ROS1 - Proto-oncogene tyrosine-protein kinase ROS

RT – Radiotherapy

SCGB3A1 – Secretoglobin family 3A member 1

SCLC - Small cell lung cancer

SEPT9 – Septin 9

SHOX2 – Short stature homeobox 2

SOX17 – Sex determining region Y box 17

SSC – Special subtype carcinoma

TET - Ten-eleven methylcytosine dioxygenase

TNBC - Triple-negative breast cancer

TP53 - Tumor Protein p53

UICC - Union for International Cancer Control

USPSTF - US Preventive Services Task Forces

VEGF – Vascular endothelial growth factor

WGA – Whole genome amplification

INTRODUCTION

EPIDEMIOLOGY

Breast cancer (BrC) is the most incident cancer in women in developed regions, followed by colorectal cancer (CRC) and lung cancer (LC) (Figure 1A) (1). Regarding cancer-related mortality, BrC is the second cause of death after LC in developed regions (1) (Figure 1B). Moreover, BrC incidence continues to increase mainly due to improvements in mammography screening (2).

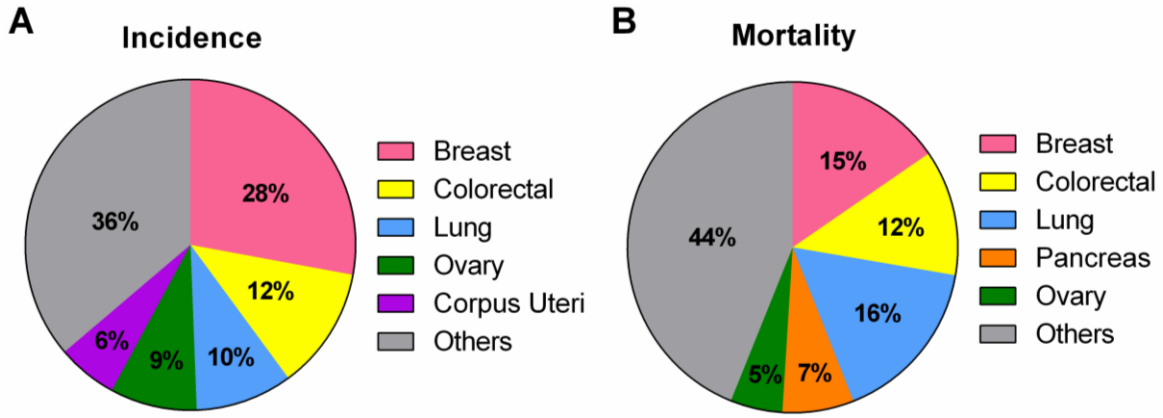

Figure 1. Estimated percentage of (A) cancer-related incidence and (B) cancer-related mortality in women in developed regions in 2012. Adapted from (1).

LC is the most common cause of cancer-related death worldwide and the first in women from developed regions (Figure 1B) (1). Nevertheless, women's incidence rates are lower when compared to men, although it increased throughout the recent years (1). A geographical pattern is verified in women's LC incidence, which can be a consequence of tobacco consumption, since LC rates are higher in Northern America and Northern Europe when compared with Western Europe and Middle Africa (3). The majority of the LC cases are diagnosed at advanced stages (approximately 75%) and associate with a 5-year survival of 10% (4).

CRC is the second most common type of cancer in women, accounting for 9.2% of women's cancer cases worldwide and the third cause of death in developed regions (Figure 1B) (1). Although 55% of CRC cases occur in more developed regions (5), CRC mortality has been decreasing in these regions due to the implementation of colonoscopy screening (1).

In Portugal, 29.4% of cancer cases in women are BrC cases, with an associated mortality rate of 16% and a prevalence of 40% (1). CRC is the second most common and deadly type of cancer, whereas LC is the 5th most incident and the 4th cause of cancer-related death (1).

BREAST CANCER

RISK FACTORS

Several risk factors are associated with increased risk for BrC development, including advanced age, exposure to female hormones, reproductive history, family history and environmental aspects (6). The risk for BrC cancer rises with age (7), although this effect is greater in premenopausal women, since the risk for developing BrC is higher than in postmenopausal (7).

Hormonal factors modulate BrC risk (8). Specifically, late menarche and an early menopause are associated with reduced BrC risk (7). Conversely, hormone replacement therapy increases the risk of BrC development in 24%, as well as the use of oral contraceptives (9). The risk for BrC development is also associated with reproductive history (6): nulliparous women have a higher relative risk of BrC development than parous women (6). Indeed, the greater the number of births, the less risk of developing BrC (9), as breastfeeding has a protective effect on BrC and the benefit is higher the longer the breastfeeding period (7).

Family history of BrC is a recognized risk factor for BrC (6, 7). Women with a first degree relative with BrC have a risk 1.5-3 times higher to develop BrC (6). BrC associated with *DNA repair associated 1 (BRCA1)* and *2* (*BRCA2*) germline mutations account for 5-10% of all BrC cases (6). Women with *BRCA* mutations have a risk up to 85% of BrC development, being generally younger and premenopausal (6). Hereditary syndromes such as Li-Fraumeni, associated with tumor protein p53 (*TP53*) mutation, and Cowden Syndrome (*PTEN* mutation) also increase the BrC risk (6). Benign breast disease, namely proliferative lesions with atypical hyperplasia are associated with a relative risk of 4.0 to 5.0 to develop BrC (6).

Environmental factors such as radiation exposure and diet have been associated with BrC. Radiation exposure by image exams [annual mammography exam associated with 86 cancers per 100,000 women (10)] and therapeutic irradiation [for example, for Hodgkin's lymphoma treatment at younger ages is associated with a 48% BrC risk (11)] increase the risk of BrC development (6, 7). Additionally, alcohol consumption and fat intake have been also associated with BrC development (7). Indeed, weight gain and obesity in post-menopausal women are associated with a 1.25 relative risk of BrC development (9).

SCREENING AND DIAGNOSIS

As stated earlier, BrC is the most diagnosed cancer in women, with increased incidence after the implementation of screening by mammography (12). Mammography is based on the usage of X-ray and allows for the identification of different densities, microcalcifications and asymmetries in the breast. For instance, multifocal fine microcalcifications are frequently associated with malignant tumors (13). Screening mammography detects BCa with 85% sensitivity and 90% specificity in elder women (14). However, the sensitivity decreases with the increase of breast density, with over 70% of the breast tumors missed in a dense breast (15).

The first clinical trial that showed the effect of screening mammography in BrC mortality started in 1963. Shapiro and its colleagues showed that mammography screening led to decreased deaths by BrC (16). In the following years several studies were conducted showing a mortality reduction ranging from 28% to 45% (17). In addition, screening mammography also allowed for tumor's early detection, and consequent reduction of morbidity related to BrC treatments (18). Although BrC screening features several benefits, it presents important disadvantages. One of the major disadvantages is the overdiagnosis (13, 19). Indeed, 11% of the cases detected in a population invited to screening that would probably not be clinically relevant in the woman's lifetime are treated (19). Another disadvantage that arises from the BrC screening are the false-positive results. These are more frequent in younger women, with ages between 40-49 years old, since the mammography sensitivity decreases in younger groups (20). False-positive results lead to extra imaging exams and eventually biopsies procedures, which can cause discomfort and anxiety to the subjects (19). Pain arising from the mammography and radiation exposure are other mentioned problems related to BrC screening (19, 21).

The BrC screening recommendations are not consensual: while mammography is the main exam used for BrC screening, the starting age the exams' interval continues to be discussed (22). European Society for Medical Oncology (ESMO) guidelines suggest that mammography screening should start at the age of 50 and end at 69, with a screening every two years (Table 1) (2). In 2015, the American Cancer Society (ACS) updated the BrC screening guidelines indicating that BrC screening should start at 45 years old (Table 1). Nevertheless, women with ages between 40 and 44 should have the possibility to perform the annually screening (23). The US Preventive Services Task Forces (USPSTF) recommends a biennial screening in women between 50 and 74 years old (Table 1) (24) However, there is insufficient evidence about the benefits of BrC screening in women with age above 75 (21). In women with higher risk of BrC, i.e., with *BRCA1/2* mutations or family

history of BrC, screening combining magnetic resonance imaging (MRI) and mammography every 6 months is currently recommended (2).

Table 1. Guidelines for BrC screening from different agencies.

Organization	Age interval	Interval between mammography
ESMO	50-69	2 years
ACS	40-44	Women should have the choice 1 year
	45-54	1 year
	≥ 55	Women should continue until is expected to live 10 more years or longer 2 years
USPSTF	40-49	Women should have the choice ½ year
	50-74	½ year

Abbreviations: ESMO - European Society for Medical Oncology; ACS - American Cancer Society; USPSTF - US Preventive Services Task Forces

The diagnosis of BrC comprises clinical examination and confirmation by histopathological analysis (2). An image-guided core needle biopsy is currently recommended for breast lesions biopsy (2). Nevertheless, a fine-needle aspiration (FNA) can be performed when a core needle biopsy cannot be obtained (2). However, although the latter is sufficient to proceed with a definitive diagnosis, it cannot distinguish invasive from non-invasive lesions and the hormone receptor status cannot be evaluated (6). Thus, when possible, a core needle biopsy is the most suitable diagnostic technique since it is more cost-effective and an adequate amount of tissue for hormone receptors status evaluation can be collected (6). FNA or core needle biopsy is recommended in suspicious lymph nodes (2).

HISTOLOGICAL SUBTYPES

Breast tumors can be divided in invasive and *in situ* carcinomas: invasive carcinomas comprise breast tissue cells' abnormal proliferation that invades through the duct wall into stroma, whereas *in situ* carcinomas are non-invasive lesions defined by the proliferation of epithelial cells confined to breast ducts or lobules (25). *In situ* lesions can be ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS), depending on cytoarchitectural features (26). Invasive ductal carcinoma (also known as ductal carcinoma NST or carcinoma of no special type) accounts for 70-80% of all breast tumors and comprises a heterogeneous group of tumors that do not display features classified as a specific histological type (25). Invasive lobular carcinoma is present in 5-15% of all BrC

patients and is considered special subtypes carcinomas (SSC), which also includes mucinous carcinoma, medullary carcinoma and invasive papillary carcinoma (25). A tumor presenting 10% to 49% of non-specialized pattern and a SSC is classified as a mixed type carcinoma (25).

STAGING

After diagnosis, the stage of each BrC patient is determined in order to evaluate the disease extension and to define the best treatment. The TNM system of American Joint Committee on Cancer (AJCC)/ Union for International Cancer Control (UICC) is the most accepted and widely used for BrC staging (27). This system comprises tumor size and extension (T), regional lymph node status (N) and presence of distant metastasis (M) (Appendix I, Table 1) (27). Different combinations of T, N and M originate 5 stages (0, I, II, III and IV), used to stratify BrC patients and to predict prognosis and treatment (Appendix I, Table 2) (25). Staging can be clinically or pathologically determined. Clinical staging is based in physical examination and imaging studies and is mainly useful in a neoadjuvant context, whereas pathological staging includes a combination of clinical data and pathological examination for BrC staging (Appendix I, Table 1) (27).

PROGNOSIS FACTORS

In addition to the staging, several biomarkers are used to predict the patient's response to therapy and prognosis. The histological grade is attributed to each tumor according to the Nottingham combined system, and a high histological grade is associated with poorly differentiated tumors and worse prognosis (25).

The estrogen receptor (ER) is a nuclear transcription factor that stimulates breast normal cells growth by the binding of estrogen. This process is particularly important in cancer development since it induces tumor growth. Seventy-five % of BrC tumors are ER-positive, being well-differentiated, less aggressive, and associated with a better prognosis (28). ER positivity is a predictor of the response to hormonal therapies (HT) with tamoxifen and aromatase inhibitors. Tamoxifen binds to the ER in BrC cells and inhibits the signaling pathways that would lead to cell growth (28). Moreover, about 65-75% of the breast tumors are progesterone receptor (PR) positive and its function is usually related to ER signaling activation (25, 28). Accordingly, PR-positive tumors are frequently ER-positive, and associate with a therapy response rate of 75-85%. On the contrary, ER^- and PR^- tumors are frequently grade 3, often recur and do not respond to HT (25).

Erb-B2 receptor tyrosine kinase 2 (ERBB2) [or human epidermal growth factor receptor 2 (HER2)] is a growth factor receptor expressed by breast epithelial cells membrane (25). Currently, it is evaluated by immunohistochemistry (IHC) and/or fluorescence in situ hybridization (FISH), being overexpressed in 15% of all BrC cases (28). Therapies targeting ERBB2 such as transtazumab and lapatinib are currently available for BrC patients, improving the patient's outcome (28).

Gene expression profiles have been emerging to assist in adjuvant treatment decision (27). Oncotype DX® (21-gene panel), MammaPrint® (70-gene panel) and PAM50 (Prosigna) (50-gene panel) are different gene expression profiles that allow for BrC tumors' classification and prognosis evaluation (29). Nonetheless, its usefulness in clinical practice is limited and therefore their implementation remains restricted, since the evidence of their using in clinical practice is limited and associated with high costs (29).

MOLECULAR SUBTYPES

BrC is a heterogeneous disease and efforts in better patients' stratification led to BrC tumors classification into molecular subtypes based on gene expression patterns (6). Different combinations of ER, PR and ERBB2 expression patterns allowed the definition of several molecular subtypes: luminal A, luminal B, ERBB2-enriched and basal-like (27). Luminal-like A and B tumors account for 70-80% of all BrC tumors. Luminal A presents high ER and PR expression levels, but low/reduced ERBB2 and Ki67 expression levels, whereas luminal B type is associated with high proliferation marker levels (Ki67) and/or ERBB2 expression (Table 2) (2). Luminal A tumors present a low number of mutations and better prognosis than luminal B tumors (2). In contrast, ERBB2-enriched tumors are positive for ERBB2 expression, but not to hormone receptors (Table 2). This molecular subtype usually displays high mutation rate, is highly proliferative and associated with poor prognosis, albeit with several available targeted therapy options (30). Basal-like BrC tumors are defined as such because express basal-cells biomarkers and are triple-negative, i.e., do not express ER, PR and ERBB2 (31). Accordingly, basal-like tumors are often referred as triple-negative breast cancer (TNBC) tumors, though not all TNBC tumors express basal-cells biomarkers (32). The basal-like subtype is frequently associated with *TP53* mutations and convey the worst prognosis among all molecular subtypes (32).

Table 2. BrC subtyping by IHC. Adapted from (2, 27).

	Luminal A	Luminal B (ERBB2-negative)	Luminal B (ERBB2-positive)	ERBB2 overexpression	Basal-like
ER	+	+	+	-	-
PR	+	-*	+/-	-	-
ERBB2	-	-	+	+	-
Ki67	-	+*	+/-	+/-	+/-

* Either PR – or Ki67 +; "+" – high expression; "-" – low expression; "+/-" – any;
Abbreviations: ER – Estrogen receptor; ERBB2 - Erb-B2 receptor tyrosine kinase 2; PR – Progesterone receptor

TREATMENT

BrC treatment decision should be provided by a multidisciplinary team including a surgeon, medical oncologist, radiation oncologist, radiologist and pathologist (2). The treatment strategy must be discussed and explained to the patient (2). Surgery, radiotherapy (RT), chemotherapy, HT and target therapy are the main modalities for BrC treatment available (33).

Surgery is the first treatment used in operable BrC tumors (2). Sixty to 80% of operable tumors allow for a breast conservation surgery with negative margins, which is associated with better cosmetic outcomes and less morbidity (2). Mastectomy is indicated for BrC patients that prefer this type of treatment and that are not indicated for breast conservation surgery, as in multifocal tumors (Figure 2) (6). Nowadays, the assessment of node-metastasis in the sentinel lymph node is a standard procedure for treatment decision making, since axillary clearance is associated with lymphedema (2).

Adjuvant RT after surgery is strongly recommended by ESMO guidelines, associating with a recurrence risk reduction by 8-15% (Figure 2) (2). Adjuvant systemic treatment might be also used based on the patients' comorbidities, preferences and risk of recurrence (2). BrC recurrence risk can be estimated by the tumor's biological characteristics (2). Indeed, low-risk luminal tumors are indicated for adjuvant therapy with HT, whereas higher risk luminal tumors (luminal B ERRB2-positive) have indication for chemotherapy, HT and targeted therapy with transtuzumab (approved for node-positive disease and for node-negative patients with tumors greater than 1 cm) (2). Furthermore, for luminal BrC, gene expression profiles such as Oncotype DX®, MammaPrint® and PAM50 can be used for adjuvant treatment decisions (2). The molecular subtypes with poorer prognosis, i.e., ERBB2-enriched and TNBC tumors have indication for chemotherapy and targeted therapy in tumors ERBB2-positive.

Moreover, neoadjuvant therapy is offered to BrC patients with locally advanced and bulky tumors that can be removed by surgery if downsized (33). Chemotherapy, HT and targeted therapy are the most commonly used neoadjuvant treatments (2). Metastatic BrC patients are usually treated in monotherapy with anthracycline or taxane chemotherapy-based regimens (34).

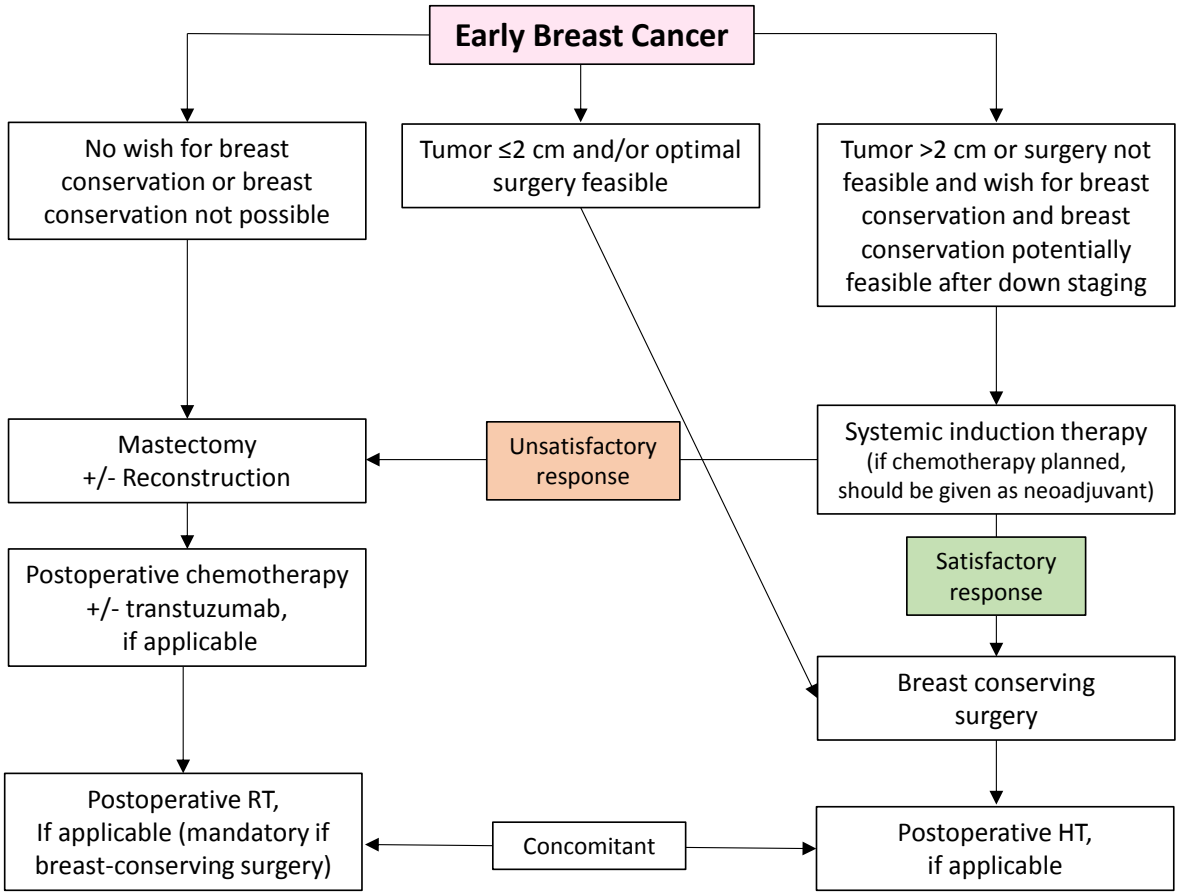

Figure 2. Early BrC treatment algorithm according to ESMO guidelines (2). Adapted from (2). Abbreviations: HT - Hormone therapy; RT - Radiotherapy

COLORECTAL CANCER

RISK FACTORS

CRC development is associated with several environmental and genetic risk factors (6). A western-type diet with high fat levels and a sedentary lifestyle increases the CRC's risk (6). Additionally, lifestyle habits such as smoking and alcohol consumption increase the risk of developing CRC in 1.11 (35) and 1.37 (for heavy drinking) (36), respectively. Furthermore, inflammatory bowel diseases history including ulcerative colitis and Crohn's disease is a risk factor for CRC development. Indeed, the risk of CRC increases with the duration and degree of inflammation (37). Conversely, fruits, vegetables, calcium and prolonged nonsteroidal anti-inflammatory drugs (NSAIDS) consumption constitute CRC protective factors (6, 38).

Hereditary syndromes account for 5-10% of all CRC cases (39). Lynch syndrome is the most common hereditary nonpolyposis colorectal cancer (HNPCC) syndrome characterized by germline mutations at the mismatch repair (MMR) genes (*MSH2*, *MLH1*, *MSH6*, *PMS2* and *EPCAM*) (40). MMR mutations usually result in microsatellite instability (MSI) (41). Its transmission is autosomal dominant and associated with a lifetime risk of developing CRC higher than 80% (40). Amsterdam and/or Bethesda criteria are useful to identify families that could carry Lynch syndrome (40). Families with Lynch syndrome are recommended to perform colonoscopy every 1-2 years (40). Hereditary polyposis syndromes such as familiar adenomatous polyposis (FAP) [Adenomatosis polyposis coli (*APC)* mutation] have 100% of penetrance and its most frequent clinical presentation is the presence of hundreds of polyps at young ages (42). MUTYH-associated polyposis, polymerase proofreading-associated polyposis, juvenile polyposis syndrome and Peutz-Jeghers syndrome are two other syndromes associated with high risk of CRC development (42).

SCREENING AND DIAGNOSIS

CRC screening has been shown to decrease the CRC incidence and mortality, mainly due to the implementation of colonoscopy and fecal occult blood test (FOBT) (6). Colonoscopy allows the visualization of the entire colon, the removal of precancerous lesions and the biopsy of a lesion present at the time of the exam (43). The evidence of colonoscopy screening arises from observational and indirect data, since there are no randomized clinical trials to demonstrate the effects of colonoscopy in CRC incidence and mortality. In a prospective study, the estimated reduction in CRC mortality in patients with

polyps removed by colonoscopy was higher than 50% (Table 3) (44). Additionally, a randomized controlled trial showed that a one-time screen with flexible sigmoidoscopy (visualization of sigmoid colon and rectum) reduced the CRC incidence by 21% and mortality by 31% (Table 3) (45). Since colonoscopy examines all the colon, better results can be expected from colonoscopy screening. As a result, several screening guidelines recommend a colonoscopy examination at the age of 50 and, if this is negative, every 10 years (39, 46). Nevertheless, colonoscopy screening involves bowel preparation, an invasive procedure and a risk of bleeding and bowel perforation (6). Furthermore, about 22% of all adenomas are missed in a colonoscopy examination, particularly in the right colon, which increases the risk of interval cancers (tumors occurring more than 6 months after a colonoscopy and prior to the next screening exam) (47).

FOBT is widely recommended for annual CRC screening due to its low invasiveness, cost and acceptable performance as a screening test (39). Two or three screenings with guaiac-based faecal occult blood testing (gFOBT), based on peroxidase-like activity of heme (6), reduced CRC mortality in 15% (Table 3) (48). However, blood presence in stool can result from dietary factors or other bowel conditions (43). Feacal immunochemical test (FIT) based on the use of antibodies for human globin increases the specificity of FOBT (43). Its performance in CRC detection is similar to guaiac-based FOBT, although it has been reported that the compliance for a FIT screening is higher, because no dietary restrictions are needed (49). Nonetheless, these tests have limited performance in detecting precancerous lesions as early lesions bleeding is uncommon (50). Multitarget DNA stool tests such as Cologuard®, that allies FOBT with *KRAS* mutation and *BMP3* and *NDGR4* methylation (51) and plasma-based biomarkers were proposed as novel screening modalities for CRC screening (52).

Table 3. Test performance of screening tests currently used for CRC screening. Adapted from (53).

	gFOBT	**FIT**	**Flexible sigmoidoscopy**	**Colonoscopy**
Sensitivity %	13-50	79	90-92	92-99
Reduction in CRC incidence %	No	Unknown	21	69
Reduction in CRC mortality %	14-16	22	31	68

Abbreviations: CRC – Colorectal Cancer; FIT - Feacal immunochemical test; gFOBT - Guaiac-based faecal occult blood testing

CRC symptoms include gastrointestinal bleeding, iron deficiency or anemia, bowel habits change, weight and appetite loss, abdominal pain and obstructive symptoms (39, 54). Acute obstruction, abdominal distention and constipation are more frequent in left colon tumors (6). A clinical evaluation with a physical examination, FOBT, and endoscopic procedure (sigmoidoscopy or colonoscopy) should be performed (39). In addition, a suspicion of metastatic disease should be confirmed by computed tomography (CT), MRI or positron emission tomography (PET) (54). The carcinoembryogenic antigen (CEA) at the diagnosis should also be evaluated, since its levels might be used as disease monitor biomarker after treatment/ follow-up (39).

MOLECULAR PATHWAYS

The majority of CRCs develop from early neoplastic lesions due to the loss of genomic and epigenomic stability (55). CRC development may result from several molecular pathways: chromosomal instability (CIN), MSI and cytosine-phosphate-guanine (CpG) island methylator phenotype (CIMP) (55). CIN accounts for 85% of the molecular pathway of sporadic CRCs and is characterized by a loss of tumor suppressor genes and a gain of oncogenes copy number (56). *APC* mutations is one of the initial key mutations involved in both sporadic CRC with CIN genotype and FAP syndrome (56). Its inactivation leads to an active WnT signaling, which stimulates cellular proliferation, migration and adhesion (56). CIN leads to further mutations, as is the case of *KRAS*, an oncogene that leads to a permanently active cellular stage and continuous growth (56, 57). This molecular pathway is the most common in the transition from adenoma to adenocarcinoma, beginning with the formation of a small adenomatous polyp that eventually evolves to a larger polyp with dysplasia and, ultimately, adenocarcinoma (58).

The MSI pathway occurs in 15% of the sporadic CRC and in more than 9% of HNPCC syndromes (57). MSI arises from the inactivation of MMR (system that repairs mismatched nucleotides in DNA replication), increasing the mutation rate of other cancer related genes in the colorectal mucosa (56). MMR's inactivation in sporadic cancers often arises from *MLH1* promoter epigenetic silencing, whereas *MLH1* and *MSH2* mutations are more common in patients with Lynch syndrome (57). Moreover, MSI CRC cancers usually present *BRAF* mutation V600E, involved with cellular responses to growth signals (57). About 15-20% of serrated adenocarcinomas have origin in sessile serrated polyps, which usually also display high levels of MSI (58).

CIMP is characterized by aberrant promoters' hypermethylation of tumor suppressor genes. It can be found in 20-30% of CRC cases (57) and CIMP-high patients display a high

rate of *BRAF* mutations. In addition to being associated with MSI, sessile serrated polyps present also high frequency of CIMP and are usually right-sided lesions (58).

HISTOPATHOLOGY AND PRECURSOR LESIONS

About 90% of CRC tumors are classified as adenocarcinomas, which originate in epithelial cells of the colorectal mucosa (59, 60). Their grading is based on the percentage of glandular formation, i.e., low-grade tumors are well or moderately differentiated tumors, whereas poorly differentiated tumors are classified as high-grade (59). Other histological variants include mucinous adenocarcinoma, with >50% of the lesion being constituted by extracellular mucin, and signet ring cell carcinoma, with prominent intracytoplasmatic mucin in more than 50% of tumor cells (59, 60). Other rare types of carcinomas comprise neuroendocrine, squamous cell, adenosquamous, and undifferentiated carcinomas (59). The majority of CRC tumors arise from precursor lesions such as adenomas (60). They can be classified as tubular, tubulovillous and villous. Adenomas with more than 1 cm in size, a villous pattern, and containing high-grade dysplasia are classified as advanced adenomas and are frequently malignant (60).

STAGING

The TNM system classification of AJCC/UICC is currently used for CRC staging (27). In CRC staging T accounts for primary tumor invasiveness: T1 involves the submucosa, T2 the invasion of *muscularis propria*, T3 the invasion of pericolorectal tissues through *muscularis propria* and T4 the invasion of visceral peritoneum or adhesion to adjacent organs or structures (Figure 3) (Appendix II, Table 3) (27). Regarding regional node status (N), at least 12 lymphatic nodes must be collected for correct staging. The N stage is attributed according to the number of nodes with metastasis (Appendix II, Table 3) (27). As staged earlier, the M parameter accounts for distant metastasis and M1 patients are classified as stage IV (Appendix II, Table 4) (27).

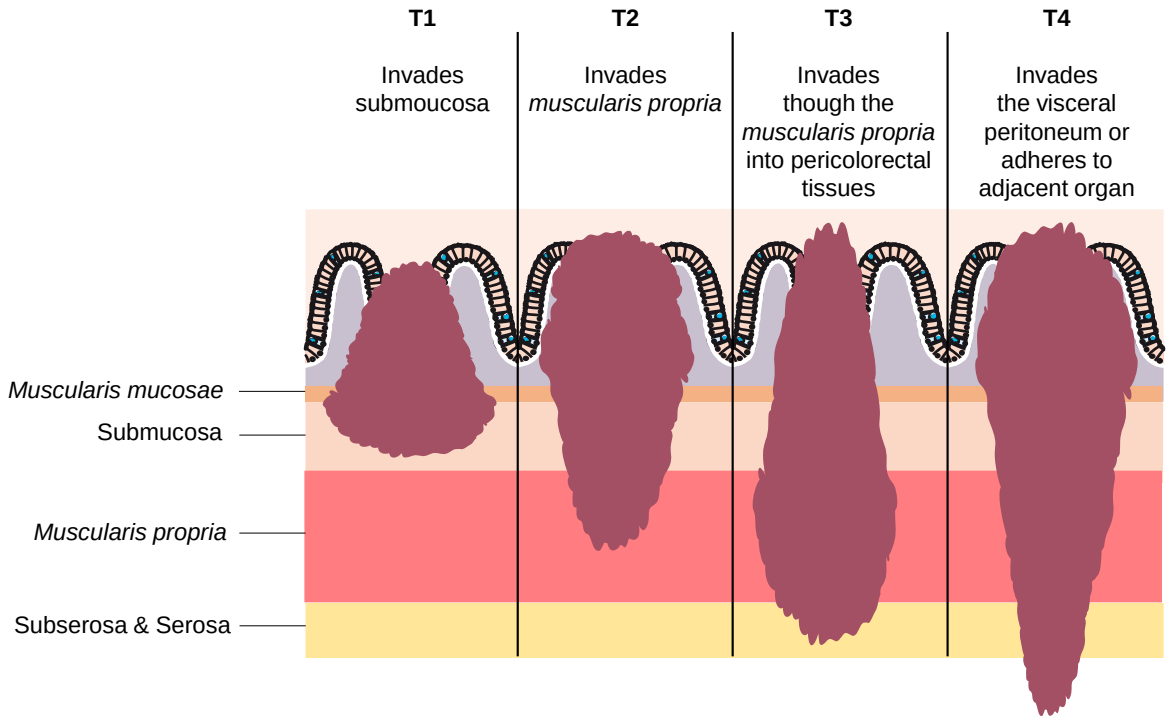

Figure 3. Definition of primary tumor (T) according to the 8th edition of AJCC Cancer Staging Manual for CRC staging. Nunes SP *unpublished*

TREATMENT

According to ESMO guidelines, the treatment of colon and rectum cancers should be decided by a multidisciplinary team according to the patient's preferences and co-morbidities (39, 54, 61). Regarding colon cancer stage 0, a local excision during an endoscopic exam should be carried out when possible. In localized disease, surgical resection is usually the first-line of treatment (Figure 4) (39). For high-risk stage II patients (lymph node sampling <12, poorly differentiated tumor, perineural invasion, presence of obstruction or perforation and pT4 stage) and stage III, the surgical resection is followed by adjuvant chemotherapy (Figure 4) (39). The recommendations for rectal cancer treatment include surgery or local RT for very early disease and chemoradiotherapy followed by surgery when possible for stages II and III (Figure 4) (61). In metastatic CRC, surgery is an option for resectable metastatic disease or for those that became resectable after tumor reduction by chemotherapy (54). For unresectable metastatic CRC, chemotherapy associated with bevacizumab [monoclonal antibody against vascular endothelial growth factor (VEGF)] is usually the first line of treatment (Figure 4) (54). Other biological agents such as aflibercept, a recombinant fusion protein that blocks VEGF, and anti-epidermal growth factor receptor (EGFR) for patients without RAS mutations are second-line options for metastatic CRC treatment (54).

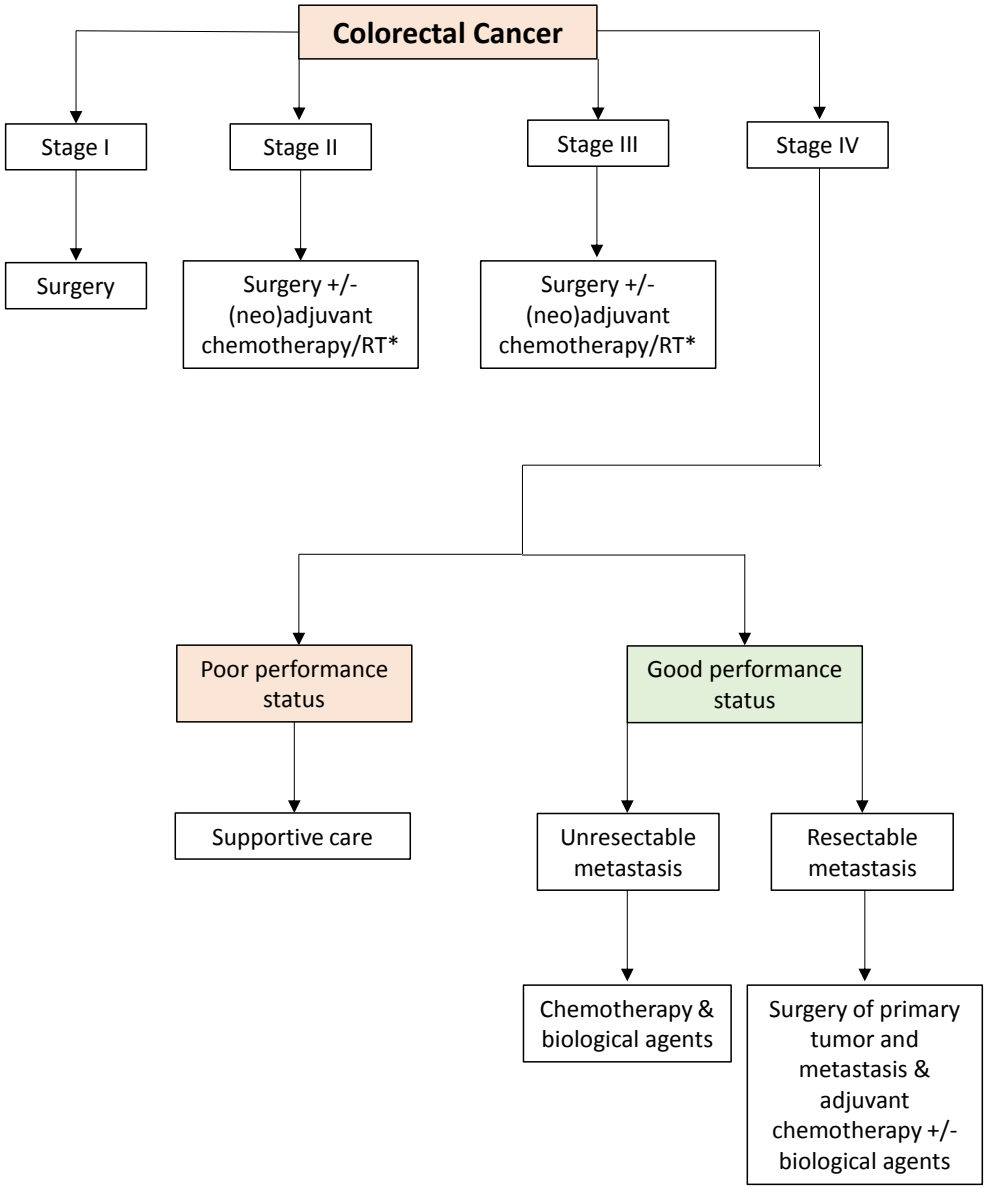

Figure 4. Simplified algorithm of CRC treatment according to ESMO guidelines (54, 61, 62). Adapted from (63). *For rectal cancer treatment.

LUNG CANCER

RISK FACTORS

Several risk factors are associated with LC development including cigarette smoking, genetic factors, asbestos and silica fibers, and exposure to ionizing radiation (6). Smoking is the most well-established risk factor of LC, since about 85% of LC cases arise from cigarette smoking (64). Smokers display a 16-fold increased risk of developing LC, which increases exponentially with the duration of smoking exposure (64). The tobacco smoke contains several carcinogens that induce malignant alterations in respiratory epithelial cells such as benzo(a)pyrene, nitrates and 4-(methylnitrosamino)-1-(13-pyridyl-1-butanone) (NNK) (6). Furthermore, smoking cessation decreases the risk of LC development, although it is never comparable to a never smoker (6). Second-hand smoking also constitutes a risk factor for LC, being associated with a 25% risk of LC development (65). Indeed, smoke carcinogens metabolites are present in body fluids of non-smokers exposed to tobacco smoke (65, 66).

Although smoking is the main risk factor, only 15% of smokers develop LC, indicating that other risk factors have also an important role in LC development (6). Family history of LC increases by 2-3 fold the risk of LC (6). Additionally, the risk of LC is higher in smokers with Li-Fraumeni syndrome (67).

Occupational and environmental exposure to asbestos and silica fibers accounts for 15% of LC cases (65), having a synergetic effect with smoking (65). Likewise, exposure to ionizing radiation such as radon and air pollution also increases the LC risk (65).

SCREENING AND DIAGNOSIS

LC is the main cause of death by cancer in women in developed regions (1), since about 75% of the lung cancers cases are diagnosed at advanced stages (68). Thus, the main goal of LC screening is to detect it in curative early stages, leading to a reduction in LC-related mortality (69).

Several studies determined the feasibility of low-dose computed tomography (LDCT) for LC screening (70-72). The National Lung Screening Trial (NLST) compared the performance of LDCT with chest X-ray in LC screening (73). A 20% decrease in LC-related mortality for the LDCT group was found, with a higher percentage of stage IA adenocarcinomas detection in the LDCT group (73). However, of the 39% positive test results in NLST, 96.4% were false-positive (73), which resulted in psychological stress and

unnecessary exams (74). Additionally, LDCT screening displays a high rate of overdiagnosis (estimated 18.5%), exposure to radiation, high costs (74), and similar clinical trials did not found a significant reduction in LC mortality with LDCT (75-77). Most recently the Dutch-Belgian Lung Cancer Screening Trial (NELSON) randomized 15 822 current or former smokers into two groups: the LDCT screening group or the control group (not offered any type of screening). The NELSON trial showed a lower false positive results rate and a higher percentage of stage I LCs detection in comparison with the NLST (78). Consequently, LC screening with LDCT is not widely implemented, however an annually LC screening with LDCT in high-risk asymptomatic adults (30 pack-year smoking history, currently smokers or have quit in less than 15 years) with ages above 55 years old is recommended (79, 80).

Patients with LC can present persistent cough, shortness of breath, chest pain and hemoptysis (6). Advanced LC may be associated with dyspnea, anorexia, weight loss and symptoms related to the metastasized organ including abdominal pain when liver, adrenal glands or pancreas are affected (81). A clinical evaluation in combination with imaging exams (chest X-ray, CT scan and PET scan) and laboratory tests should be performed for tumor extension assessment (Figure 5) (82). Moreover, a tumor's sample should be collected either by bronchoscopy, sputum cytology or fine-needle aspiration, depending on the tumor's location (Figure 5) (82).

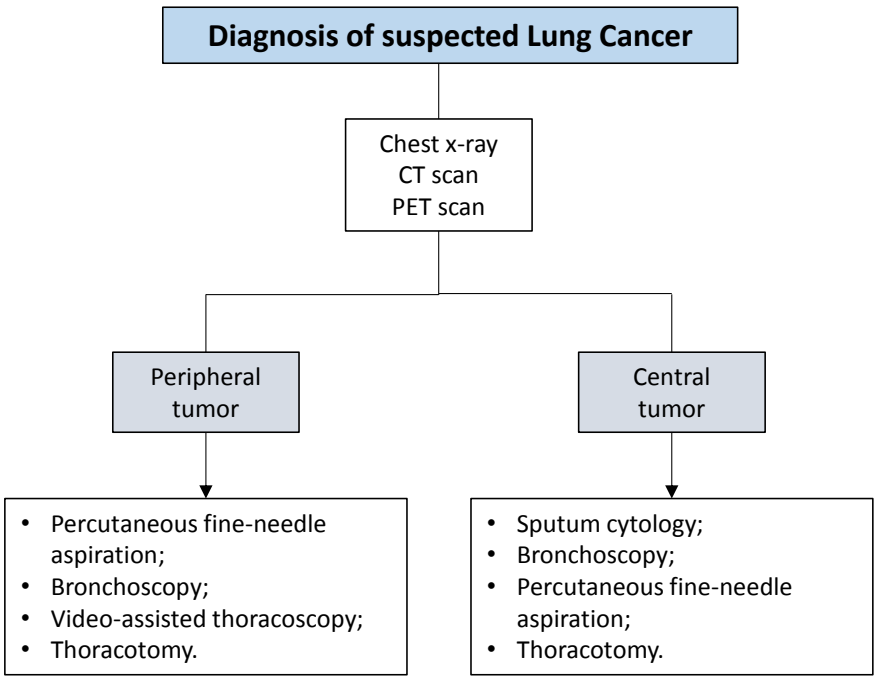

Figure 5. Scheme of the diagnostic procedures depending on the lesion location in lung. Adapted from (6) Abbreviations: CT - Computed tomography; PET - Positron emission tomography.

HISTOLOGICAL SUBTYPES

LC histological major subtypes comprise the non-small cell lung cancer (NSCLC) (adenocarcinoma, squamous cell carcinoma and large-cell carcinoma), that accounts for 80% of all LC cases, and the small cell lung cancer (SCLC) (81). Adenocarcinoma has emerged as the main LC subtype (>40% of LC cases) due cigarettes' manufacturing modifications (81). Specifically, the introduction filters and new blends lead to a puff volume increase, which caused a shift from squamous cell carcinoma and SCLC (central deposition of the smoke) to adenocarcinoma (more peripheral deposition of tobacco smoke) (81). LC adenocarcinoma usually arises from peripheral airways and displays glandular differentiation with mucin production or pneumocyte marker expression (81). The prognosis is strongly related with the stage, however never-smokers and female patients usually display a better prognosis (81). Squamous cell carcinoma that histologically exhibits keratinization and/or intercellular bridges, constitutes the second most frequent LC subtype (81). These tumors usually have a superficial growth and spread to adjacent structures (81).

SCLC is the subtype most associated with smoking and accounts for 13% of all LC cases (81). It arises in a central location with a rapid growing and metastization, representing the most aggressive LC subtype (6). Histologically, SCLC presents densely packed small tumor cells with a diffuse growth pattern and a high degree of mitosis (81). As expected, SCLC displays a low 2-year survival rate (about 10%) and the worst prognosis of all LC subtypes (81). Carcinoid tumors are also neuroendocrine tumors that represent 1% of all LC tumors (83). They frequently arise from central airways and can be divided in carcinoid typical or atypical, depending on the grade (typical, low-grade and atypical, intermediate-grade) (83). Atypical carcinoid has a poorer prognosis than typical carcinoid, the latter presenting a 5-year survival rate of 90% (81).

STAGING

As for BrC and CRC, the TNM system is used for LC staging determined by clinical or pathological evaluations (27). The clinical staging uses information before the treatment, whereas pathological staging is assessed after surgery in order to decide the adjuvant treatment (27). The parameter T accounts for the tumor size and invasion. Furthermore, the regional node status N assesses the nodal chains affected and M the presence of distant metastasis (Appendix III) (27).

TREATMENT

The treatment of LC includes surgery, chemotherapy, RT and target agents in the presence of *EGFR* or anaplastic lymphoma kinase (*ALK*) mutations (82, 84). For stages I and II, surgery with complete resection of the lesion and, at least, six lymphatic nodes is the first line treatment recommended (82). Adjuvant chemotherapy is recommended for resected NSCLC stages II and III and for all stages of SCLC after surgery (82, 84). Postoperative RT is recommended in patients with the resection margins positive and with lymph node metastasis (82). Stereotactic RT is preferred in NSCLC stage I patients with comorbidities or that cannot be operated for other reasons (82). In advanced LC, surgery followed by adjuvant chemotherapy or chemoradiotherapy should be applied in resectable tumors, whereas chemoradiotherapy is the first recommended line of treatment for unresectable LC (induction chemotherapy followed by RT) (82). Chemotherapy is used as the first treatment for metastatic SCLC and NSCLC without mutations on *EGFR*, proto-oncogene tyrosine-protein kinase ROS (*ROS1*) or *ALK* genes, (85), whereas target therapy is used for NSCLC with *EGFR*, *ROS1* or *ALK* mutations (85). Recently, immunotherapy with programmed death-ligand 1 (PD-L1) inhibitors was approved for metastatic NSCLC patients (86).

Several mutations in proto-oncogenes were identified as potential targets for LC therapy. *EGFR* is a transmembrane tyrosine kinase that leads to cell proliferation when activated. In the presence of *EGFR* mutations, the signaling pathway is constitutively activated (6). *EGFR* mutations are more frequent in never and light-smokers and occur in 17% of adenocarcinomas (87). The presence of *EGFR* mutations is the main predictor of response to *EGFR*-target therapies such as gefitinib, erlotinib and afatinib (85). Nonetheless, 50% of LC patients have disease progression due to *EGFR* acquired mutations and a second-line of *EGFR* inhibitors can be offered (85). In adenocarcinomas, *ALK* and *ROS1* rearrangements predict response to crizotinib, a targeted agent that blocks the signaling by tyrosine kinases (85, 88).

Thus, detection of cancer early stages increases the likelihood of a successful treatment, leading to a potential cure and low-rate of recurrences. Although current screening strategies are beneficial, they have significant limitations, comprising risk of overdiagnosis/overtreatment, invasiveness and high cost, entailing low compliance and suboptimal specificity, requiring further testing and increasing suspects' anxiety (19, 50). Hence, development of better pre-screening methods, which might perfect selection to invasive/costly screening tests is mandatory.

EPIGENETICS

Epigenetics, firstly mentioned by C. Waddington in 1942, can be defined as heritable alterations in gene expression without changing the DNA sequence (89). The epigenetic regulation is essential to normal cell mechanisms, namely embryonic development, imprinting and tissue differentiation (90). The main epigenetic mechanisms include DNA methylation, histone post-translational modification, histone variants and chromatin remodeling complexes (Figure 6) (91). The study of the epigenome increased the understanding of cancer, since deregulation of epigenetic mechanisms are implicated in cancer development (89, 90).

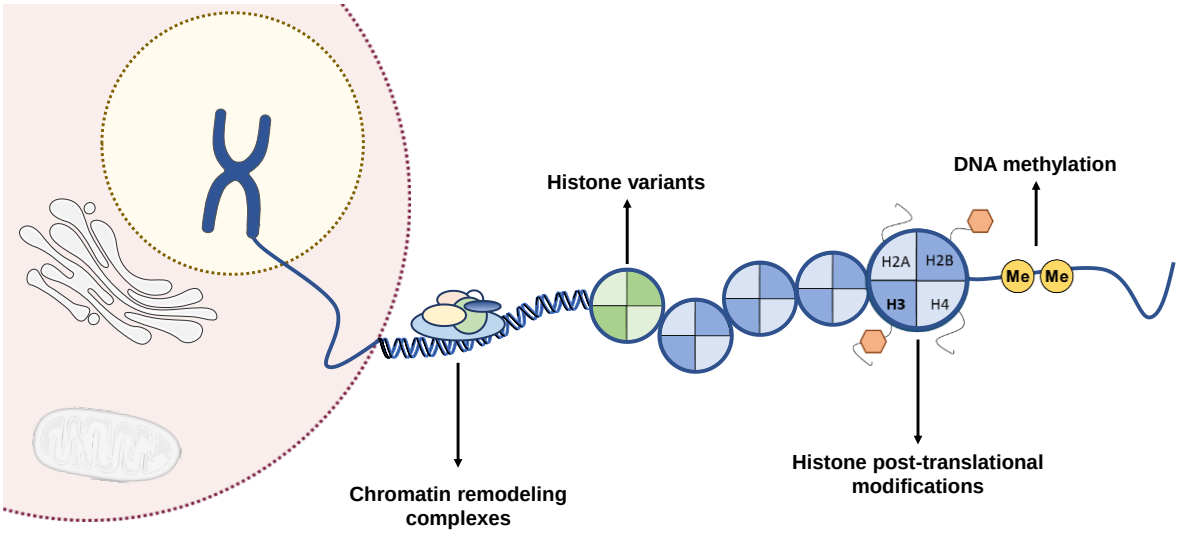

Figure 6. Four main epigenetic mechanisms involved in gene expression regulation. DNA methylation consists in an addition of a methyl group in cytosine present in a cytosine-phosphate-guanine (CpG) dinucleotide. Histone post-translational modifications refer to alterations in histone tails such as methylation, acetylation, phosphorylation and ubiquitination that regulate gene expression. Histone variants differ few amino acids from canonical histones and regulate chromatin remodeling and histone post-translational modifications. Chromatin remodeling complexes regulate the nucleosome structure by removing, relocate and shifting histones. (Kindly provided by Lameirinhas A. *unpublished*)

DNA METHYLATION

DNA methylation is the most investigated epigenetic alteration that consists in a covalent addition of a methyl group at the 5-position carbon of cytosine present in a CpG dinucleotide (91). In normal mammalian cells, CpG dinucleotides are usually methylated. Nevertheless, unmethylated CpGs are frequently found in CpG islands (sequences with 200-500 bases with more than 50% of CpG dinucleotides in content) (90). About 50% of CpG islands are present in gene's promoter regions, and their methylation is associated with transcription repression (90). On the other hand, methylation can also occur in the gene body that, conversely, is associated with transcriptional activation (90). DNA methylation

can also be found in CpG island shores (2 kb areas upstream of a CpG island with CpG dinucleotides, but in lower frequency than CpG islands). CpG island shores' methylation is also associated with transcriptional repression and tend to be tissue-specific (90). DNA methylation can induce the binding of transcriptional repressors or impede the binding of transcriptional factors, resulting in gene transcription inhibition (90). In fact, a family of methyl-CpG-binding proteins (MBPs) intervenes in the tumor suppressor genes' silencing by binding to the methylation CpGs and recruiting histone modification enzymes to establish histone post-translation modifications to further sustain transcriptional repression (92).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs), namely DNMT3a and DNMT3b that catalyze *de novo* DNA methylation during embryonic development, establishing tissue-specific DNA methylation, and DNMT1 that preferably maintains the DNA methylation patterns already existing (90). X chromosome inactivation in female mammals, silencing of repetitive centromeric sequences and transposons along with genomic imprinting are cells' main DNA methylation mechanisms in order to maintain genomic stability (90). DNA demethylation is performed by ten-eleven methylcytosine dioxygenase (TET) enzymes, that catalyze the conversion of 5-methylcytosine to 5-hydroxymethylated cytosine, maintaining CpG islands' unmethylated state (92).

Generally, global hypomethylation is observed in cancer epigenome, which contributes to overexpression of proto-oncogenes, mutation rates increase and loss of imprinting, resulting in genomic instability (93). Indeed, a decrease from 80% to 40-60% in methylation levels from normal cells to cancer cells is observed (93). Simultaneously the promoter's hypermethylation at 5' regions of tumor suppressor genes is also a frequent event in cancer cells (91). This alteration results in tumor suppressor genes' inactivation, namely genes that inhibit invasion, metastasis and angiogenesis, and DNA repair genes. Typically, 5-10% of CpG island promoters are methylated in cancer (93).

Importantly, cancer-related genes methylation has been proposed as a potential biomarker for cancer detection and monitoring (94) due to several features: (i) it is a stable alteration; (ii) it is frequent and arises early in cancer development; (iii) its detection can be easily performed with quick and reliable techniques as sodium-bisulfite modification and quantitative methylation-specific PCR (qMSP) (iv) it can be detected in several biological samples including formalin-fixed paraffin-embedded tissues and several body fluids (sputum, serum/plasma, urine, stool) (94, 95). Indeed, the detection of DNA methylation in serum/plasma has shown to be promising in several cancers detection using a minimally-invasive strategy (95).

LIQUID BIOSPIES

As earlier mentioned, tissue biopsy remains the standard method for cancer diagnosis. Nevertheless, it has been associated with several disadvantages: it may not represent the tumor heterogeneity and it does not allow to assess the treatment efficacy and detection of early recurrences or residual disease (96, 97). Therefore, a minimally-invasive method that allows cancer detection and diagnosis in an early stage and patients follow-up is essential. Recently, the concept of liquid biopsy has emerged using biomarker analyses of blood. Circulating cell-free tumor DNA (ccfDNA), circulating tumor cells (CTCs), circulating cell-free RNA and exosomes are the main forms of liquid biopsy described so far (98, 99).

CcfDNA was firstly reported in 1948 in blood of healthy donors (100) and after in cancer patients (101). CcfDNA fragments can be 70-200 base-pairs long in healthy controls, while ccfDNA in tumor patients can range from 150 to 1000 base-pairs, representing 0.01-50% of all ccfDNA (99). The concentration of ccfDNA in blood also varies between healthy controls (0-100 ng/mL) and cancer patients (0-1000 ng/mL) (102). CcfDNA has a short half-life ranging from 15 minutes to hours, being removed by the liver or kidney (102). Several studies showed that tumor ccfDNA can have various origins: (i) released from living tumor cells (103); (ii) apoptotic or necrotic tumor cells (104); (iii) CTCs (105) (Figure 7).

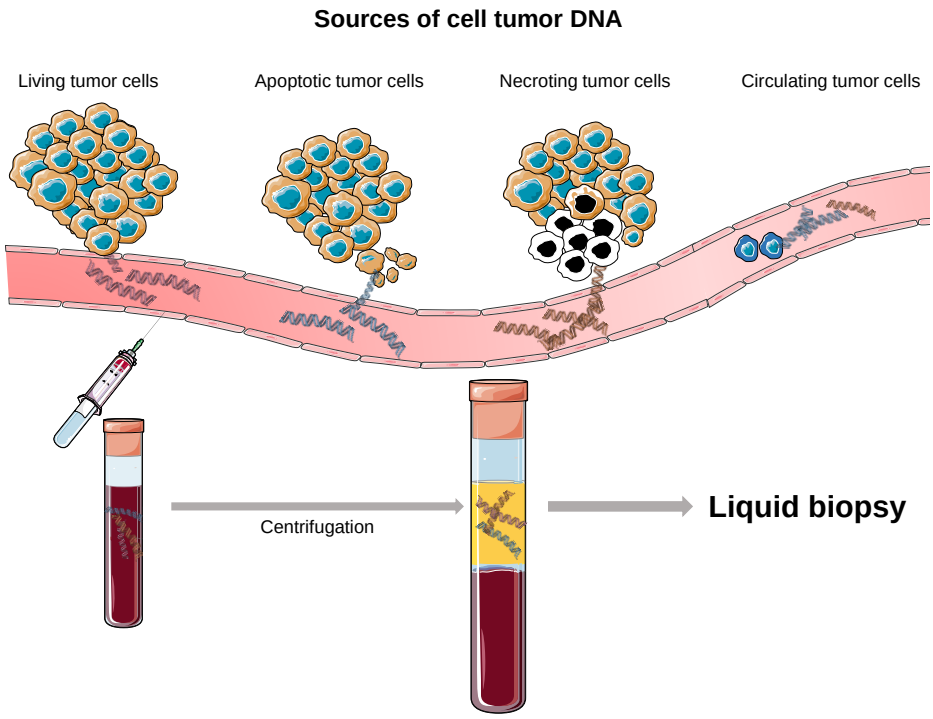

Figure 7. CcfDNA liquid biopsy. Tumor DNA can have several origins, namely living tumor cells, apoptotic or necrotic tumor cells and circulating tumor cells (CTCs). Blood can be collected, the ccfDNA can be extracted from serum/plasma and analyzed in a context of a liquid biopsy. Nunes SP *unpublished.*

It has been shown that ccfDNA contains alterations present in the tumor itself, namely mutations (106) and methylation alterations (107). The detection of tumor-specific DNA methylation in ccfDNA constitutes a promising approach for cancer detection and monitoring, even in early lesions, which has been demonstrated by already approved tests such as Epi proColon® and Epi proLung® for CRC and LC detection in plasma, respectively (108, 109). Tumor ccfDNA for patient monitoring, namely a test for *EGFR* mutations, is already approved in NSCLCs liquid biopsies for disease monitoring and treatment response prediction (110).

DNA METHYLATION-BASED BIOMARKERS

Several methylated genes have been proposed as tumor biomarkers for BrC, CRC and LC detection. Nevertheless, the sensitivity for cancer detection of one methylated gene in ccfDNA is limited, hence several studies attempted to assemble gene-methylation panels to increase the test sensitivity (111-114).

APC and Ras association domain family 1 isoform A (*RASSF1A*) are widely mentioned in several panels for BrC, CRC and LC detection (111, 115, 116) (Table 4, Table 5, Table 6). *APC*, as a tumor suppressor gene, has a crucial role in Wnt signaling pathway, since its silencing by mutation or hypermethylation leads to an accumulation of β-catenin with consequent activation of Wnt signaling pathway (117). In addition, *APC* has been implicated in DNA repair, cytoskeleton regulation and apoptosis (117). *RASSF1A* is a tumor suppressor gene involved in apoptosis, cell-cycle progression and cell adhesion (118). Although *APC* and *RASSF1A* individually display a low sensitivity, their specificity for BrC detection is higher than 95% (111).

Furthermore, retinoic acid receptor beta 2 (*RARβ2*) is commonly hypermethylated in cancer patients and mentioned in panels for cancer detection (111, 119). Its main function is to regulate the epithelial growth by inducing growth inhibition and apoptosis induction in the presence of retinoic acid (120). *RARβ2* promoter's hypermethylation can be found in 40% of LC patients and BrC cell lines (120, 121). Additionally, a four-gene panel with *RARβ2, p16*^{INK4a}, 6-methylguanine DNA methyltransferase (*MGMT*), and *APC* hypermethylation detected 75% of CRC patients in stool samples (122).

Sex determining region Y box 17 (*SOX17*) has been recognized as an antagonist of Wnt signaling pathway (123). *SOX17* promoter's methylation is reported in ccfDNA and CTCs of BrC patients (124), CRC cell lines and tumors (125), and plasma of NSCLC patients (123). Indeed, a gene panel with *CDO1*, *TAC1* and *SOX17* displayed a sensitivity of 93% for LC detection (Table 6) (126).

Table 4. Previously published methylation panels for BrC detection in ccfDNA liquid biopsies.

Breast Cancer					
Gene Panels	Sensitivity (%)	Specificity (%)	Specimen type	Methods	References
hMLH1 HOXD13 P16 PCDHGB7 RASSF1A SFN	79	72	Serum	qMSP	(112)
GSTP1 RARβ2 RASSF1A	22	93	Serum	One-step MSP	(127)
FKBP4 KIF1A MAL OGDHL VGF	49	80	Plasma	qMSP	(128)
APC DAP-kinase RASSF1A	94	100	Serum	MSP	(129)
DKK3 ITIH5 RASSF1A	67	69	Serum	qMSP	(130)
APC GSTP1 RARβ2 RASSF1A	62	87	Plasma	qMSP	(111)
ATM RASSF1A	36	100	Plasma	qMSP	(131)
ESR1 14-3-3-σ	81	55	Serum	qMSP	(132)
APC ESR1 RASSF1A	53	84	Serum	qMSP	(133)
DAPKI RASSF1A	96	92	Serum	MSP	(134)
RARβ2 RASSF1A SCGB3A1 TWIST	98	82	Serum	qMSP	(135)
CDH1 RASSF1A	76	90	Serum	MSP	(136)

Abbreviations: MSP – methylation-specific PCR; qMSP – quantitative methylation-specific PCR

Secretoglobin family 3A member 1 (*SCGB3A1*) promoter's hypermethylation is described as the main mechanism for its loss in BrC tumors and cells (137). Additionally, respective hypermethylation activates AKT signaling pathway in NSCLC cell lines (138). Moreover, *SCGB3A1* has been associated with increased apoptosis and inhibition of cell cycle reentry, migration and invasion (137). *SCGB3A1* hypermethylation was already mentioned as a potential biomarker for BrC detection in a four-gene panel with a sensitivity of 98% (Table 4) (135).

MGMT gene encodes a DNA repair protein that eliminates aberrant DNA bases exposed to alkylating and methylating agents (139). Methylation of *MGMT* promoter has been associated with several cancers including glioblastomas, CRC and BrC (139-141).

Fork-head box A1 (*FOXA1*) is a transcriptional factor involved in the embryonic development and tissue differentiation in prostate, breast, gastro-intestinal tract and lung (142). *FOXA1* was shown to inhibit cell growth and to induce the expression of cell-cycle inhibitors including cyclin G2 (143). *FOXA1* activity is related to ERα, since nearly 50% ER-target genes are regulated by *FOXA1* (144). Moreover, loss of *FOXA1* or *FOXA2* in pancreatic cancer cells is associated with epithelial-to-mesenchymal transition (145).

Septin 9 (*SEPT9*) belongs to a GTP binding protein family and is involved in normal cell mechanisms including division of cytoplasm, membrane reconstruction and cell polarization (146). In CRC, the expression of *SEPT9* decreases with the progression from adenoma to invasive carcinoma (147). *SEPT9* promoter's methylation levels are currently used in several tests in ccfDNA extracted from plasma as a non-invasive biomarker for CRC detection (148, 149). As an example, Epi proColon® showed a 68% sensitivity and 80% specificity in plasma samples from CRC patients (Table 5) (149).

Short stature homeobox 2 (*SHOX2*) is a transcription factor involved in skeleton development and heart development (150). An association between *SHOX2* promoter's methylation and LC has been established, being explored as a possible LC biomarker (151, 152). Indeed, recently a two-gene panel based on *SHOX2* and *PTGER4* methylation levels was approved in ccfDNA in Europe (109). The gene-panel detected 85% of the LC patients with a specificity of 50% (Table 6) (109).

Thus, the assessment of aberrant DNA methylation constitutes a valuable biomarker in ccfDNA extracted from plasma/serum, being a potential non-invasive strategy for early cancer detection.

Table 5. Previously published methylation panels for CRC detection in ccfDNA liquid biopsies.

Colorectal Cancer					
Gene Panels	Sensitivity (%)	Specificity (%)	Specimen type	Methods	References
SEPT9 **Epi proColon®**	68	80	Plasma	qMSP	(149)
^{m}BMP3 ^{m}NDRG4 ***KRAS*** **mutation** **Fecal hemoglobin** **Cologuard®**	92	87	Stool	Multiplex QuARTS	(51)
^{m}Vimentin ***ColoSure™***	41	95	Stool	MSP	(153)
ALX4 ***SEPT9***	71	95	Plasma	qMSP	(154)
ALX4 SEPT9 ***TMEFF2***	81	90	Plasma	Multiplex qMSP	(155)
NPY PENK ***WIF1***	87	80	Serum	Multiplex qMSP	(113)
APC MGMT ***RASSF2A Wif-1***	87	92	Plasma	MSP	(115)
BCAT1 ***IKZF1***	77	92	Plasma	Two-step qMSP	(156)
GATA5 ***SFRP2***	81	55	Plasma	MSP	(157)
EFHD1 ***PPP1R3C***	90	64	Plasma	MSP	(158)
HLTF ***hMLH1***	57	90	Serum	qMSP	(159)

Abbreviations: MSP – methylation-specific PCR; qMSP- quantitative methylation-specific PCR

Table 6. Previously published methylation panels for LC detection in ccfDNA liquid biopsies.

Lung Cancer					
Gene Panels	Sensitivity (%)	Specificity (%)	Specimen type	Methods	References
***PTGER4 SHOX2* Epi proLung®**	85	50	Plasma	Multiplex qMSP	(109)
p16^{INK4A} CDH13	39	100	Serum	MSP	(160)
PRDM14 RASSF1A 3OST2[a]	83	76	Sputum	qMSP	(161)
APC CDH13 DLEC1 KLK10 RASSF1A	84	74	Plasma	MSP	(116)
CDKN2A/p16 DAPK GSTP1 RARβ2 RASSF1A	73	71	Serum	MSP	(119)
APC CDH1 AIM1 MGMT RASSF1A	75	73	Serum	qMSP	(114)
AJAP1 CDO1 HOXA9 MARCH11 PTGDR UNCX[a]	72	71	Serum	qMSP	(162)
CDO1 TAC1 SOX17	93	62	Plasma	qMSP	(126)
DCC Kif1a NISCH RARβ2	73	71	Plasma	qMSP	(163)
DAPK MGMT p16/INK4a RARβ2 RASSF1A	50	85	Serum	MSP	(164)
CDH13 CDKN2A/p16 FHIT RARβ2 RASSF1A ZMYND10	73	82	Plasma	Two-step MSP	(165)

[a]Only stage I patients; Abbreviations: MSP – methylation-specific PCR; qMSP- quantitative methylation-specific PCR

AIMS

Despite efforts in early cancer detection, especially in BrC and CRC screening programs, BrC, CRC and LC remain the most incident and deadly cancers in women in developed regions. Hence, advances in detection of these 3 major cancers are needed, specifically in pre-screening methods, which might select patients to invasive/costly screening tests, avoiding overdiagnosis and unnecessary exams.

Because aberrant promoter methylation of cancer-related genes is frequent in the earliest steps of cancer development, DNA methylation has been proposed as a promising biomarker for cancer detection. Moreover, this is a stable alteration that can be detected in serum/plasma's ccfDNA and easily quantified by methylation-specific PCR methods.

Thus, the main aim of this study was to develop a sensitive and specific methylation-based test in ccfDNA liquid biopsies for simultaneous BrC, CRC and LC detection in women. For that:

- Nine genes (*APC, FOXA1, MGMT, RARβ2, RASSF1A, SCGB3A1*, *SEPT9*, *SHOX2* and *SOX17*) identified as methylated in cancer samples were selected from literature and previous data from our group (166-168);
- Promoter's methylation levels of 9 genes were assessed by multiplex qMSP in ccfDNA extracted from plasma samples from female cancer patients (BrC, CRC and LC) and asymptomatic controls (AC);
- The association between the gene methylation levels and clinicopathological parameters was evaluated;
- A gene-panel with the best combination of sensitivity and specificity for cancer detection ("PanCancer" panel) was determined;
- A gene-panel suitable to discriminate the 3 cancer types ("CancerType" panel) was identified.

MATERIAL AND METHODS

CLINICAL SAMPLES

PATIENTS AND SAMPLES COLLECTION

Blood samples were collected from female patients primarily diagnosed with BrC, CRC and LC at the time of diagnosis prior to any treatment from 2015 to 2018 at the Portuguese Oncology Institute of Porto, Portugal. Additionally, for control purposes, blood samples from female healthy donors with ages over 45 years old at the Portuguese Oncology of Porto were collected between 2016 and 2018 (Table 7). Relevant clinical data was collected from clinical records and displayed in a data base for analysis purposes.

This study was approved by the institutional review board (Comissão de Ética para a Saúde – CES 120/2015) of Portuguese Oncology Institute of Porto, Portugal. All patients and healthy donors enrolled in this study provided written informed consent, in accordance with the Declaration of Helsinki ethical principles.

Table 7. Number of plasma samples of the BrC, CRC, LC patients and ACs.

Type	Number of plasma samples
BrC	108
CRC	72
LC	73
AC	103

SAMPLES PROCESSING

Blood samples were collected into two or three EDTA tubes from each individual patient or healthy donor and was centrifuged at 2,000 rpm for 10 min. at 4°C. Plasma was collected and immediately frozen at -80°C.

CELL-FREE DNA EXTRACTION

The ccfDNA was extracted from ~3 mL of plasma using QIAamp MinElute ccfDNA (Qiagen, Germany), according to manufacturers' recommendations. Firstly, 60 µL of magnetic bead suspension, 110 µL of proteinase K, and 300 µL of bead binding buffer were added to the plasma in a 15 mL tube. After a 10 min. incubation period, the tubes were placed in a magnetic rack and the supernatant was discarded when the solution was clear. Then, 200 µL of bead elution buffer were added and the mixture was transferred to a bead elution tube and incubated for 5 min. with shaking at 300 rpm. The bead elution tube was placed in a 2 mL magnetic rack and the supernatant was transferred to a new tube. 300 µL

of buffer ACB were added to the samples and the mixture was briefly vortexed and centrifuged. Next, the samples were transferred into a QIAamp UCP MinElute column and centrifuged at 6,000 x g for 1 minute. The column was washed 2 times with 500 µL of buffer ACW2, followed by a centrifugation at 14,000 rpm for 3 min.. Subsequently, the QIAamp UCP MinElute column was transferred to a 1.5 mL elution tube and the samples were incubated at 56ºC for 3 min. with the lid open. Ultimately, ccfDNA was eluted in 20 µL of sterile distilled water, incubated for 2 min. and then centrifuged at 14,000 rpm for 1 min.. All the steps were performed at room temperature. The extracted ccfDNA was stored at -20°C until further use.

SODIUM-BISULFITE MODIFICATION

Sodium-bisulfite modification constitutes a gold-standard technique for methylation studies (169). It is based on consecutive chemical reactions (sulphonation, desulphonation and deamination) that lead to the conversion of all unmethylated cytosine residues to uracil residues, whereas methylated cytosines remain 5-methylcytosines (170) (Figure 8). The converted DNA can be used to methylation studies with PCR and sequencing, since it is possible to design primers and probes specifically for the modified DNA sequence (169).

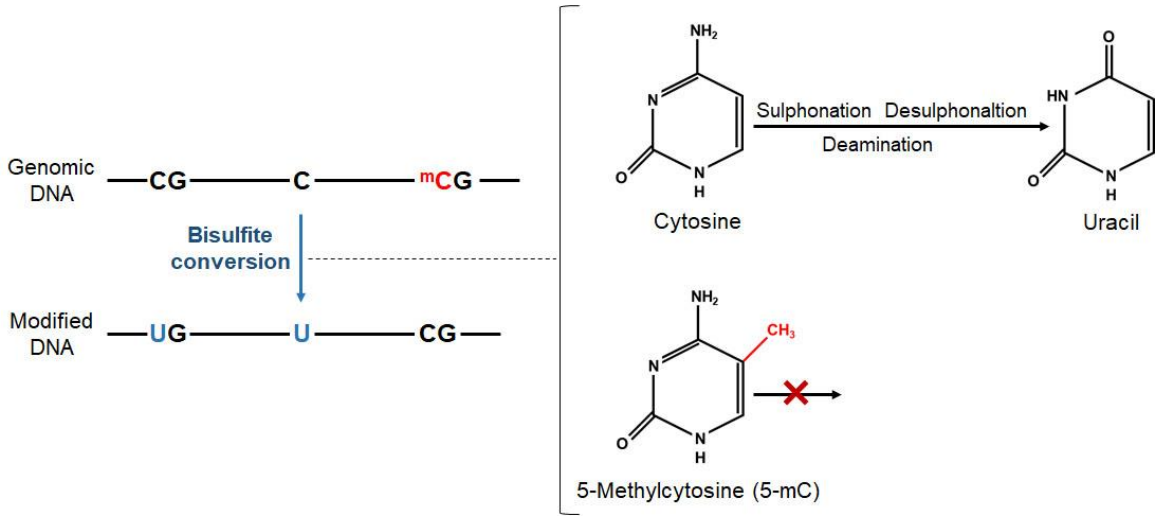

Figure 8. Sodium-bisulfite modification of DNA. The unmethylated cytosines are converted to uracil, whereas methylated cytosines remain unchanged. Abbreviations: C – cytosine residue; ^{m}C – methylated cytosine residue; G – guanine residue; U – uracil residue; 5-mC – 5-methylcytosine. (Kindly provided by A. Lameirinhas *unpublished*)

All ccfDNA samples were modified using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) according to the manufacturer's instructions. The first step consisted in adding 130 µL of CT conversion reagent solution to 20 µL extracted ccfDNA of each sample. Next, each sample was incubated at 98ºC for 10 min. for DNA denaturation and at 64ºC for 180 min. in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). After, the samples and 600 µL of M-Binding buffer were added to Zymo-Spin™ IC column and were incubated at room temperature for 10 min.. The columns were centrifuged at 10,000 rpm for 30 seconds. After the addition of 100 µL of M-Wash buffer and further centrifugation, 200 µL of M-Desulphonation buffer were added to the column followed by a 20 min. incubation and a 10,000 rpm centrifugation for 30 seconds. Then, the column was washed two times with 200 µL of M-Wash buffer and two centrifugations at 10,000 rpm for 30 seconds. The columns were placed in a 1.5 mL safe-lock tube where 10 µL of sterile distilled water were added to elute the bisulfite-converted DNA. After a 5 min. incubation at room temperature, the columns were centrifuged at 12,000 rpm for 30 seconds. This process was repeated twice. One µg of CpGenome™ Universal Methylated DNA (Millipore, USA) was modified using the protocol above mentioned and was eluted in 30 µL of sterile distilled water. The bisulfite-converted DNA was stored at -80°C until further use.

WHOLE GENOME AMPLIFICATION

The ccfDNA is present in low quantity and quality in plasma/serum samples, and the sodium-bisulfite modification leads to further DNA degradation (171). The whole genome amplification (WGA) of bisulfite-converted DNA is a method that amplifies samples with low DNA quantity, yielding high DNA amounts for methylation analysis (172). A multiple displacement amplification (MDA) method was used for WGA in this study. MDA is based on an isothermal reaction using a DNA polymerase with exonuclease proofreading activity to decrease potential amplification biases (173).

WGA of sodium-bisulfite modified ccfDNA was carried out using the EpiTect Whole Bisulfitome Kit (Qiagen, Germany) according to the manufacturer's recommendations. Briefly, 10 µL of modified DNA were mixed with 30 µL of EpiTect amplification master mix that contained 29 µL of EpiTect WBA reaction buffer and 1 µL REPLI-g Midi DNA polymerase. Then, the samples were incubated in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA) at 28°C for 8 hours and 95°C for 5 min. for polymerase inactivation. The amplified DNA was diluted in 25 µL of sterile distilled water, in a final volume of 65 µL, and stored at -20°C until further use.

NUCLEIC ACID QUANTIFICATION

The DNA concentration was measured using the Qubit fluorometric method, in which a fluorescent dye binds specifically to a target, such as a double- or single-stranded DNA (174).

The extracted ccfDNA and amplified DNA were quantified using Qubit dsDNA HS Assay Kit (Invitrogen, California, USA), and the sodium-bisulfite converted DNA was quantified with Qubit ssDNA Assay Kit (Invitrogen, California, USA). Firstly, a working solution containing 199 µL of Qubit dsDNA HS Buffer/Qubit ssDNA Buffer and 1 µL of Qubit dsDNA HS Reagent/Qubit ssDNA Reagent per sample was prepared. Then, 199 µL of the working solution were added to 1 µL of the DNA sample. The DNA concentration was determined using Qubit 2 Fluorometer (Invitrogen, California, USA) following the manufacturers' recommendations.

MULTIPLEX QUANTITATIVE METHYLATION SPECIFIC PCR

The nine genes promoters' methylation levels (*APC*, *FOXA1*, *MGMT*, *RARβ2*, *RASSF1A*, *SCGB3A1*, *SEPT9*, *SHOX2* and SOX17) were assessed by multiplex qMSP. Primers and probes specifically designed to the modified gene sequence plus the fluorochromes and quenchers used for each probe are listed in Table 8. *β–Actin* was used as a reference gene to normalize the DNA quantity of each sample (167).

Table 8. Primers and probes sequences with respective fluorochrome and quencher.

Gene		Sequences
β-Actin	Primers	F – 5' TGG TGA TGG AGG AGG TTT AGT AAG T 3'
		R – 5' ACC AAT AAA ACC TAC TCC TCC CTT AA 3'
	Probe	5' Cy5 – ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA – QSY 3'
APC	Primers	F – 5' TGT GTT TTA TTG CGG AGT GC 3'
		R – 5' CAC ATA TCG ATC ACG TAC GC 3'
	Probe	5' VIC – CAATCGACGAACTCCCGAC – MGB 3'
FOXA1	Primers	F – 5' CGA CGT TAA GAC GTT TAA GC 3'
		R – 5' CGC TCA ACG TAA ACA TCT TAC 3'
	Probe	5' FAM -ATA TAC GAA TAA AAC GAC TTA ACG – MGB 3'
MGMT	Primers	F – 5' TTT CGA CGT TCG TAG GTT TTC GC 3'
		R – 5' GCA CTC TTC CGA AAA CGA AAC G 3'

	Probe	5’ VIC – TGC GTA TCG TTT GCG – MGB 3’
RARβ2	Primers	F – 5’ TCG AGA ACG CGA GCG ATT 3’
		R – 5’ GAC CAA TCC AAC CGA AAC 3’
	Probe	5’ HEX – CTT ACA AAA AAC CTT CCG AAT ACG TTC CGA – Iowa Black RQ-Sp 3’
RASSF1A	Primers	F – 5’ AGC GAA GTA CGG GTT TAA TC 3’
		R – 5’ ACA CGC TCC AACC GA ATA 3’
	Probe	5’ NED – CGG GAG TTG GTA TTC GTT GGG CG – QSY 3’
SCGB3A1	Primers	F – 5’ GTA CGG TCG TGA GCG GAG C 3’
		R – 5’ GAA ACT TCT TAT ACC CGA TCC TC 3’
	Probe	5’ FAM – GCC GAC CTC GCC CGC GCT CCT AAA – Iowa Black RQ-Sp 3’
SEPT9	Primers	F – 5’ TTA GTT AGC GCG TAG GGT TC 3’
		R – 5’ ACC TTC GAA ATC CGA AAT AA 3’
	Probe	5’ NED – GCG TTA ACC GCG AAA TCC GAC ATA ATA ACT – QSY 3’
SHOX2	Primers	F – 5’ ATT CGT ATT TGG TCG CGT AC 3’
		R – 5’ CTA CTA CGA CCG CCA CTA CC 3’
	Probe	5’ FAM – CAA CGT AAC GAA CG – MGB 3’
SOX17	Primers	F – 5’ GAT CGG TTC GTT TTC GTC G 3’
		R – 5’ GCC CGT ATT CTA ACC TAT CG 3’
	Probe	5’ Cy5 – ACC GAC CTA ATA ACA CTA CGA ACG C – Iowa Black RQ-Sp 3’

The multiplex qMSP assays were carried out in 96-well plates (GRiSP, Portugal) using a 7500 Sequence Detector (Applied Biosystems, Perkin Elmer, CA, USA). The multiplex gene combinations used are displayed in Table 9.

Table 9. Gene combinations for multiplex qMSP.

Combination 1	Combination 2	Combination 3
β-Actin	*MGMT*	*RARβ2*
APC	*SEPT9*	*SCGB3A1*
FOXA1	*SHOX2*	*SOX17*
RASSF1A	—	—

For gene combinations 1 and 2, per well: 10 µL Xpert Fast Probe (GRiSP, Portugal), 0.8 µL of a mix with forward and reverse primers (10 µM) (Sigma-Aldrich, Germany), 0.1 µL of TaqMan probe (10 µM) (Applied Biosystems), 6 µL of amplified DNA and sterile distilled water (B.Braun, Melsungen, Germany) were added, totaling 20 µL of reaction volume. For combination 3, per well: 10 µL Xpert Fast Probe, 0.3 µL of each Primer PCR Custom Assay (BioRad, United States), 6 µL of amplified DNA and sterile distilled water were added, in a final volume of 20 µL.

The PCR program used was the following: 1 cycle at 95ºC for 3 min.; 50 cycles at 95ºC for 5 seconds and 60ºC for 30 seconds. All samples were run in triplicate and three negative template controls (WGA-amplified water) were included in each plate. In addition, CpGenome™ Universal Methylated DNA (Merck Millipore, Germany) underwent WGA and was used as a template to six serial dilutions (5x factor dilution), which were included in each plate. These serial dilutions were used to generate a standard curve, which allowed the relative quantification and PCR efficiency evaluation. All plates presented efficiency values above 90%.

The relative DNA methylation levels for each gene in each sample was calculated using the following formula:

$$Methylation\ level = \frac{Target\ gene}{\beta - Actin} \times 1000$$

STATISTICAL ANALYSIS

Non-parametric tests were used to compare methylation levels of each gene promoter between cases and respective controls and to evaluate associations with clinicopathological features. Mann-Whitney U test was used for comparisons between two groups and Kruskall-Wallis test for three or more groups, followed by Mann-Whitney U test with Bonferroni's correction for pairwise comparisons. Correlations between methylation levels and age were assessed by Spearman nonparametric correlation test. A *p* value <0.05 was considered statistically significant.

For each gene, samples were categorized as methylated or unmethylated based on cut-off value determined using Youden's J index (value combining highest sensitivity and specificity), through ROC curve analysis (175). Validity estimates [sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy] were calculated to assess biomarker performance (Table 10). A positive result was considered

when a sample was classified as methylated and negative when unmethylated. Gene panels were constructed to maximize detection performance, considering a positive result whenever at least one gene promoter was methylated. The validity estimates for "PanCancer" panel were determined by assembling BrC + CRC + LC samples (n=253) *vs*. AC samples (n=103). For "CancerType" panel, the cut-offs were determined based on the following combinations: (i) for *SCGB3A1*, BrC samples (n=108) *vs*. CRC + LC samples (n=145); (ii) *SEPT9*, CRC samples (n=72) *vs*. BrC + LC samples (n=181); (iii) *SOX17*, LC samples (n=73) *vs*. BrC + CRC samples (n=180). A multiple ROC curves via resampling analysis was performed in order to calculate the validity estimates for "PanCancer" and "CancerType" panels. Briefly, the samples were randomly divided in a training (70%) and validation (30%) sets. The cut-off value comprising the highest sensitivity and specificity was estimated in the training set and the validity estimates were calculated in the validation set using that cut-off. This procedure was repeated 1,000 times, and the mean of the sensitivities and specificities was calculated. These calculations were performed using R v3.4.4. Two-tailed *p*-values calculation and other ROC curve analyses were performed using a computer assisted program (SPSS Version 24.0, Chicago, IL, USA). Graphics were assembled with GraphPad 6 Prism (GraphPad Software, La Jolla, CA, USA).

Table 10. Formulas for biomarkers performance calculations.

Tumor *vs*. Control			Sensitivity (%)	(C/E) ×100
	Tumor	Control	Specificity (%)	(B/F)×100
< cut-off	A	B	PPV (%)	(C/(C+D))×100
> cut-off	C	D	NPV (%)	(B/(A+B))×100
Total	E	F	Accuracy (%)	[(C+B)/(E+F)]

Abbreviations: PPV – Positive Predictive Value; NPV – Negative Predictive Value.

RESULTS

CLINICAL AND PATHOLOGICAL DATA

This study included 253 female patients with BrC (n=108), CRC (n=72) or LC (n=73) and 103 female healthy donors (AC), which served as controls. Detailed clinical and pathological characterization is provided in Table 11. Globally, the median age of cancer patients significantly differed from that of controls (p<0.0001), and, thus, correlations between age and gene promoter methylation levels were assessed stratifying for ACs and cancer patients. Although *SOX17* promoter's methylation levels correlated with controls' age (R=0.225, p=0.009), this was not observed in cancer patients and no other correlations were disclosed.

Table 11. Clinical and pathological features of BrC, CRC and LC patients and ACs enrolled in this study.

Clinicopathological features	**AC**	**Cancer Patients**
Number	103	253
Age median (range)	52 (45-65)	63 (29-93)
		Breast cancer
Histological Type		
Invasive Carcinoma, no special type (NST)	n.a.	80
Invasive lobular carcinoma		12
Ductal carcinoma i*n situ*		7
Other invasive carcinoma subtypes[a]		9
Estrogen Receptor Status[b]		
Positive	n.a.	91
Negative		15
Progesterone Receptor Status[c]		
Positive	n.a.	81
Negative		24
Molecular Subtype[d]		
Luminal	n.a.	90
ERBB2 overexpression		4
TNBC		10
Primary Tumor (T)		
Tis	n.a.	7
T1 / T2		95
T3 / T4		6
Regional lymph node (N)		
N0	n.a.	65
N+		43
Distant metastasis (M)		
M0	n.a.	103
M1		3

Clinical Stage		
0	n.a.	7
I / II		88
III / IV		13
		Colorectal cancer
Histological Type		
Premalignant Lesions[e]	n.a.	3
Adenocarcinoma (all subtypes)		68
Neuroendocrine carcinoma		1
Tumor location		
Proximal colon	n.a.	23
Distal colon		30
Rectum		19
Primary tumor (T)[b]		
Tis	n.a.	3
T1 / T2		18
T3 / T4		49
Regional lymph node (N)[b]		
N0	n.a.	37
N+		33
Distant metastasis (M)		
M0	n.a.	66
M1		6
Clinical Stage		
0	n.a.	3
I / II		34
III / IV		35
		Lung cancer
Histological Type		
Non-small cell lung carcinoma (NSCLC)		
Adenocarcinoma	n.a.	56
Other NSCLC subtypes[f]		8
Small-cell lung carcinoma (SCLC)		8
Carcinoid tumor		1
Primary Tumor (T)[d]		
T1	n.a.	18
T2 / T3 / T4		51
Regional lymph node (N)[g]		
N0	n.a.	27
N+		45
Distant metastasis (M)		
M0	n.a.	36
M1		37

Clinical Stage		
I / II	n.a.	21
III / IV		52

[a]Includes medullary, mucinous and mixed type carcinoma (invasive carcinoma, NST and micropapillary carcinoma); [b]Not determined in 2 cases; [c]No information available in 3 cases; [d]No information available in 4 cases; [e]Includes tubulovillous adenoma with high-grade dysplasia and intramucosal adenocarcinoma; [f]Includes squamous cell carcinoma and large-cell neuroendocrine carcinoma; [g]Not possible to determine in 1 case; AC, Asymptomatic Control; n.a. – not applicable

GENE PROMOTER METHYLATION LEVELS IN ccfDNA

The gene promoter methylation levels of *APC*, *FOXA1*, *MGMT*, *RARβ2*, *RASSF1A*, *SCGB3A1*, *SEPT9*, *SHOX2* and *SOX17* were evaluated in ccfDNA extracted from cancer patients and ACs plasma samples. *APC*, *FOXA1*, *RASSF1A* and *SCGB3A1* promoters depicted significantly higher methylation levels in BrC patients than in controls (p<0.0001, p=0.0063, p=0.0003 and p=0.0245, respectively) (Figure 9). Nonetheless, no significant differences were found for *MGMT*, *RARβ2*, *SHOX2*, *SEPT9* and *SOX17* (p=0.984, p=0.611, p=0.090, p=0.168 and p=0.815, respectively) (Figure 9).

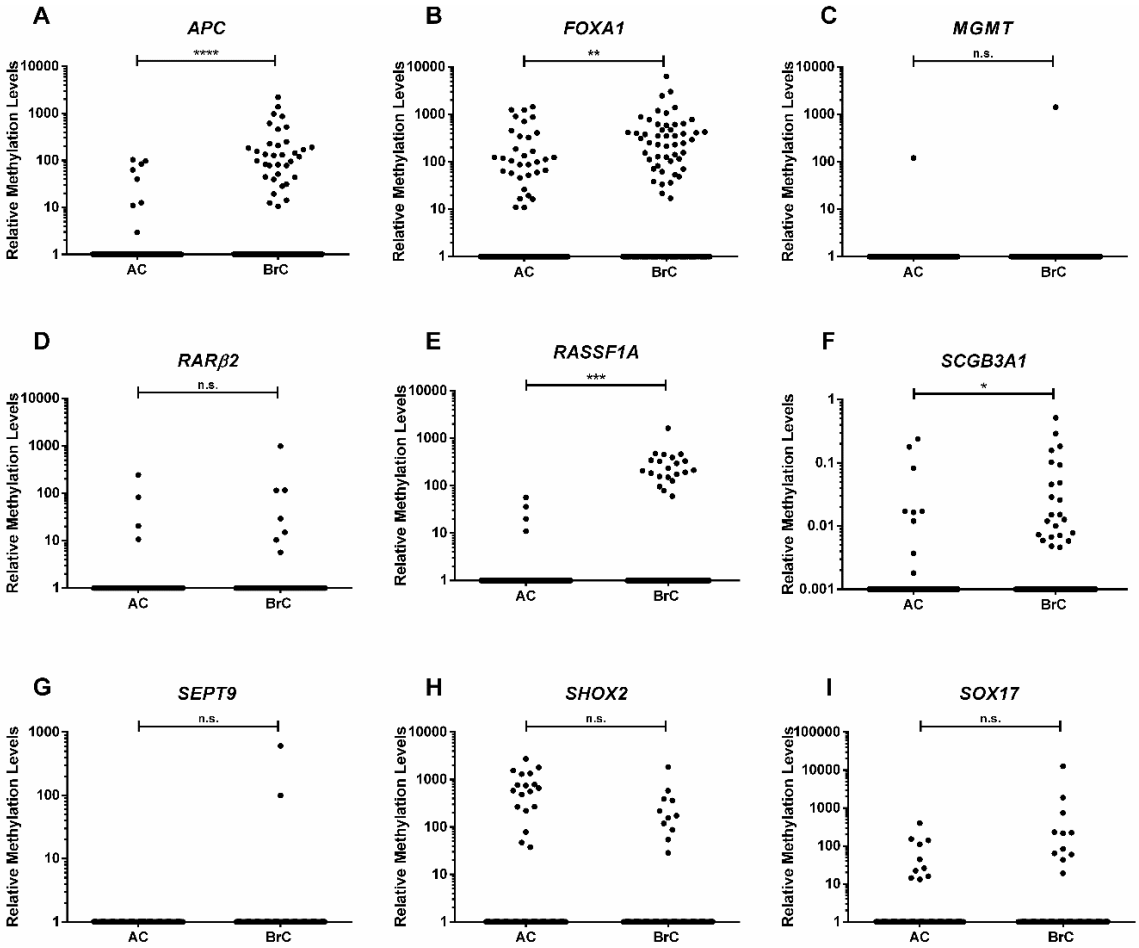

Figure 9. Scatter plot of the distribution of (A) *APC*, (B) *FOXA1*, (C) *MGMT*, (D) *RARβ2*, (E) *RASSF1A*, (F) *SCGB3A1*, (G) *SEPT9*, (H) *SHOX2* and (I) *SOX17* promoters' methylation levels in BrC patients (n=108) and ACs (n=103). Mann Whitney Test, n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In CRC patients, *APC*, *FOXA1*, *RARβ2*, *RASSF1A*, *SCGB3A1*, *SEPT9* and *SOX17* methylation levels were significantly higher than in controls (p=0.005, p<0.0001, p=0.009, p=0.012, p=0.003, p=0.001 and p=0.007, respectively) (Figure 10), although no differences were apparent for *MGMT* and *SHOX2* methylation levels (p=0.074 and p=0.077, respectively) (Figure 10).

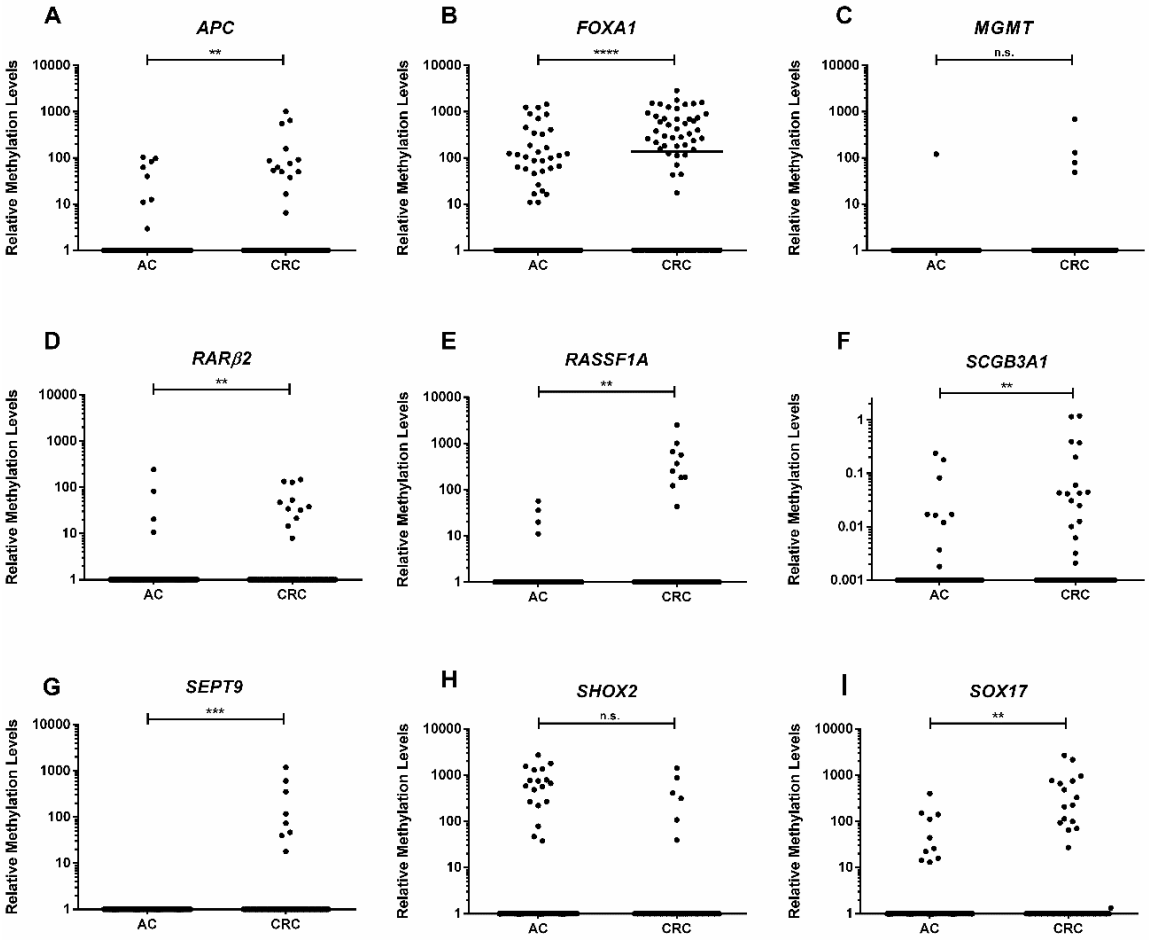

Figure 10. Scatter plot of the distribution of (A) *APC*, (B) *FOXA1*, (C) *MGMT*, (D) *RARβ2*, (E) *RASSF1A*, (F) *SCGB3A1*, (G) *SEPT9*, (H) *SHOX2* and (I) *SOX17* promoters' methylation levels between CRC patients (n=72) and ACs (n=103). Mann Whitney Test, n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Black horizontal line represents the methylation levels' median.

Concerning LC, significantly higher methylation levels compared to controls were disclosed for *APC*, *FOXA1*, *RARβ2*, *RASSF1A* and *SOX17* (p<0.0001 for all genes), whereas *MGMT*, *SCGB3A1* and *SHOX2* did not display differences between LC patients and ACs (*p*=0.400, *p*=0.084 and *p*=0.214, respectively) (Figure 11).

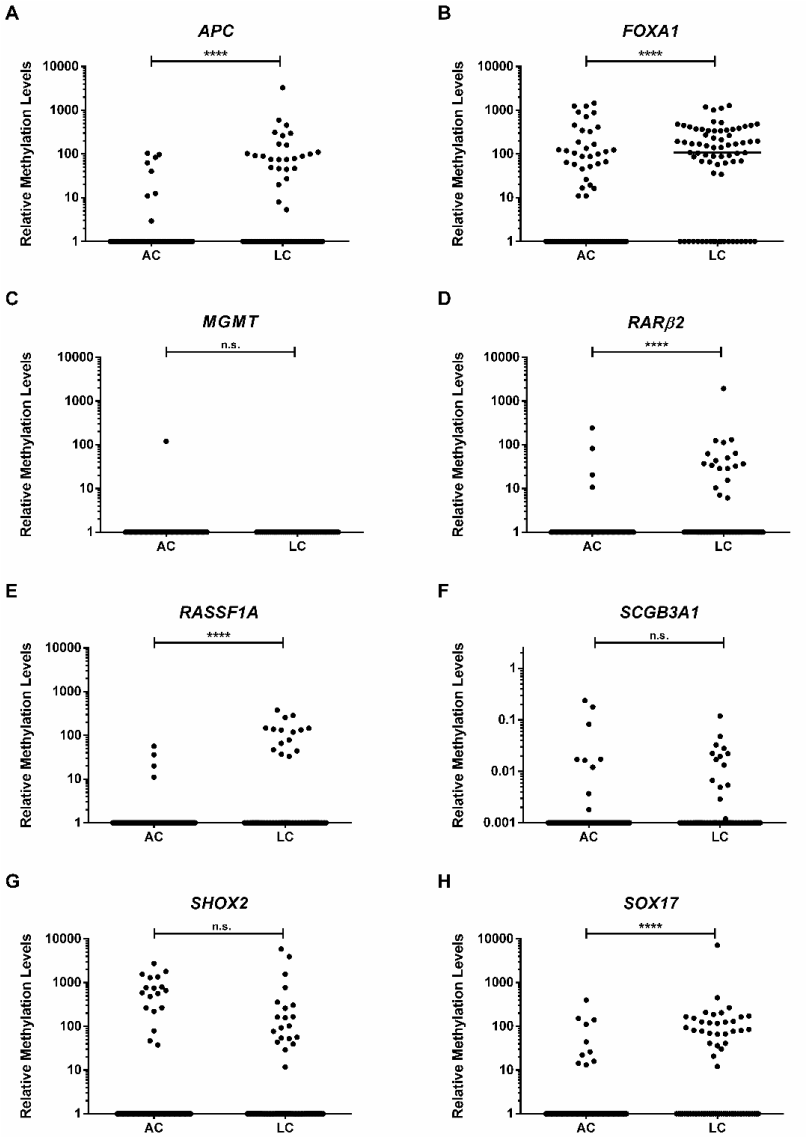

Figure 11. Scatter plot of the distribution of (A) *APC*, (B) *FOXA1*, (C) *MGMT*, (D) *RARβ2*, (E) *RASSF1A*, (F) *SCGB3A1*, (G) *SHOX2* and (H) *SOX17* promoters' methylation levels between LC patients (n=73) and ACs (n=103). *SEPT9* only presented zero values for AC and LC patients. Mann Whitney Test, n.s. *p*>0.05, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Black horizontal line represents the methylation levels' median.

ASSOCIATION BETWEEN PROMOTERS' METHYLATION LEVELS AND CLINICOPATHOLOGICAL FEATURES

Methylation levels of tested gene promoters were associated with several clinicopathological features. Specifically, in BrC patients, *RASSF1A* methylation levels significantly differed between PR+ and PR- tumors (p=0.031) (Figure 12A), whereas *RARβ2* promoter methylation levels were higher in node-positive than in node-negative BrC patients (p=0.008) (Figure 12B).

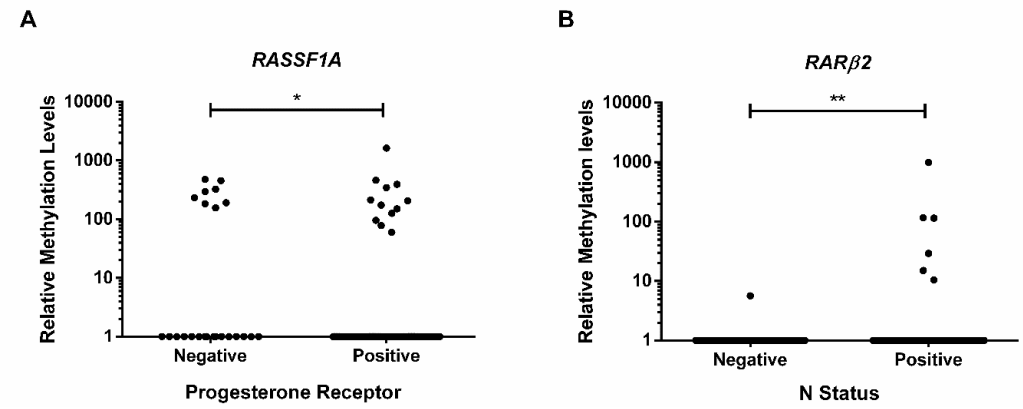

Figure 12. Scatter plot of (A) *RASSF1A* promoter's methylation levels between positive and negative Progesterone Receptor (Negative n=24, Positive n=94) and (B) *RARβ2* promoter's methylation levels between node-negative and node-positive BrC patients (Negative n=65, Positive n=43). Mann Whitney Test, *p<0.05, **p<0.01.

Moreover, in CRC patients, *SEPT9* promoter methylation levels were significantly higher in patients with stage IV or distant metastatic disease (M1) (p<0.0001, in all comparisons) (Figure 13B). Similar results were depicted for *APC*, *SHOX2* and *SOX17* promoter methylation in metastatic *vs*. non-metastatic CRC patients (p=0.0276, p=0.0107 and p=0.0242, respectively), although no differences were found for stage (Figure 13).

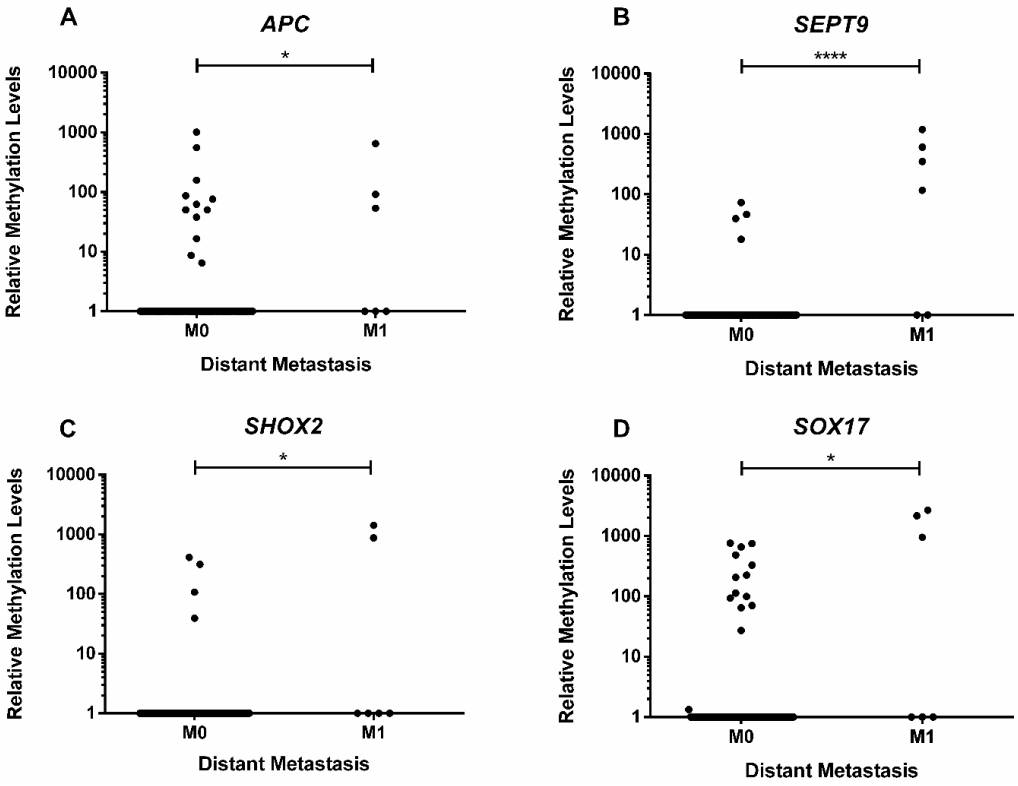

Figure 13. Scatter plot of (A) *APC*, (B) *SEPT9*, (C) *SHOX2* and (D) *SOX17* promoter's methylation levels between metastatic CRC patients (M1) and non-metastatic CRC patients (M0) (M0 n=66, M1 n=6). Mann Whitney Test, *p<0.05, ****p<0.0001.

Concerning LC, significantly higher *APC* and *RARβ2* promoter methylation levels were apparent in SCLC patients than those with adenocarcinoma (p=0.005 and p=0.035, respectively) (Figure 14A, Figure 14B). Moreover, node-positive LC patients displayed higher *RASSF1A* methylation levels than node-negative LC patients (p=0.018, Figure 14C), whereas higher *SOX17* promoter methylation was observed in patients with systemic metastization (p=0.029) (Figure 14D).

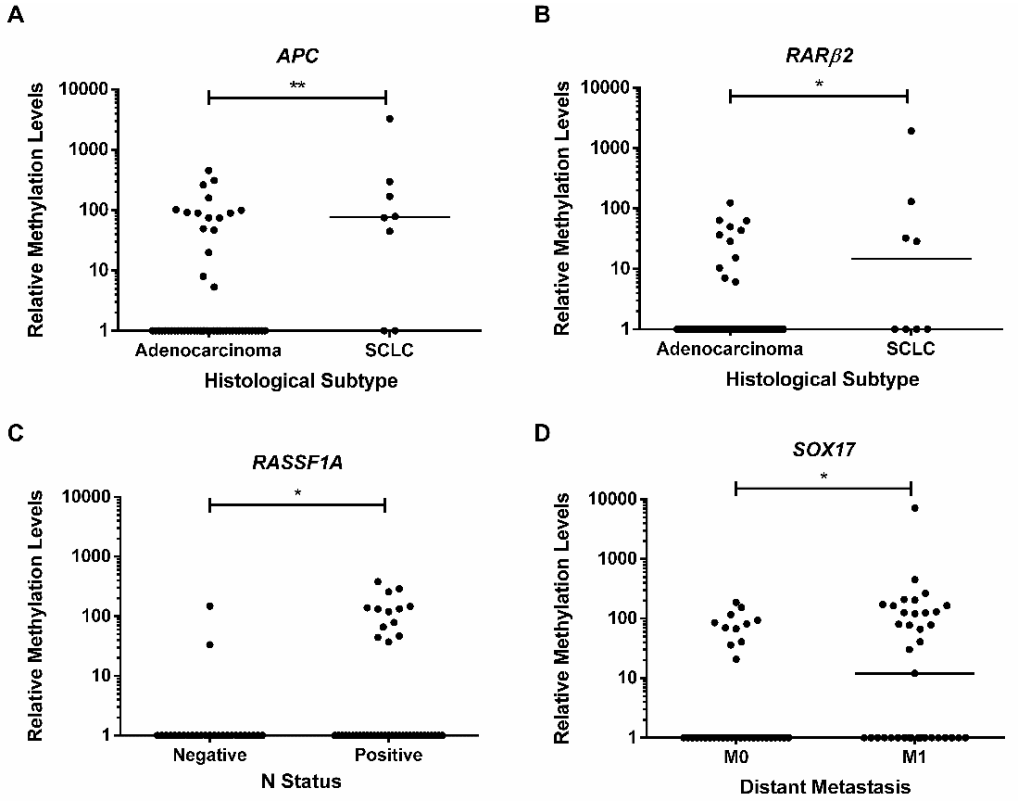

Figure 14. Scatter plot of (A) *APC* and (B) *RARβ2* promoters' methylation levels for Histological Subtype (Adenocarcinoma n=55, SCLC n=8), (C) *RASSF1A* promoter's methylation levels for Regional Node (N) status (Negative=27, Positive=45) and (D) *SOX17* promoter's methylation levels between metastatic LC patients (M1) and non-metastatic LC patients (M0) (M0 n=36, M1 n=37). Mann Whitney Test, **p*<0.05, ***p*<0.01. Black horizontal line represents the methylation levels' median.

BIOMARKER PERFORMANCE OF ccfDNA

Gene promoters disclosing significantly higher methylation levels in cancer patients *vs.* controls were selected for assessment of BrC, CRC or LC detection performance in ccfDNA. *APC*, *FOXA1* and *RASSF1A* individually depicted sensitivity over 20% and specificity greater than 70%, for all cancers. *FOXA1* displayed the highest sensitivity (39% for BrC, 50% for CRC and 73% for LC) (Table 12). Overall *RASSF1A* disclosed the highest specificity (over 98%) for all three cancer types and *SEPT9* displayed 100% specificity for CRC detection (Table 12). *SCGB3A1* detected BrC and CRC with over 20% sensitivity (Table 12), whereas *RARβ2* and *SOX17* displayed specificity higher than 90% for CRC and LC detection (Table 12).

Table 12. Biomarker performance of each promoter's gene methylation for BrC, CRC and LC detection in ccfDNA.

Genes	Cut-off value	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
Breast Cancer						
APC	6.710	32.41	94.17	85.37	57.06	62.56
FOXA1	68.01	38.89	79.61	66.67	55.41	58.77
RASSF1A	24.88	19.44	100.0	100.0	54.21	58.77
SCGB3A1	4.130×10^{-3}	21.30	92.23	74.19	52.78	55.92
Colorectal Cancer						
APC	4.711	20.83	94.17	71.43	62.99	64.00
FOXA1	142.4	50.00	88.35	75.00	71.65	72.57
RARβ2	3.949	16.67	95.15	70.59	62.03	62.86
RASSF1A	39.47	13.89	99.03	90.91	62.20	64.00
SCGB3A1	6.291×10^{-5}	26.39	90.29	65.52	63.70	64.00
SEPT9	8.973	11.11	100.0	100.0	61.68	63.43
SOX17	0.6633	23.61	90.29	62.96	62.84	62.86
Lung Cancer						
APC	4.115	35.62	94.17	81.25	67.36	69.89
FOXA1	30.12	72.60	73.79	66.25	79.17	73.30
RARβ2	3.056	24.66	95.15	78.26	64.05	65.91
RASSF1A	26.57	21.92	98.06	88.89	63.92	66.48
SOX17	28.16	38.36	95.15	84.85	68.53	71.59

Abbreviations: PPV - Positive Predictive Value; NPV - Negative Predictive Value; n.a. - not applicable

Since *APC*, *FOXA1* and *RASSF1A* were biomarkers common to BrC, CRC and LC, they were further tested as gene panel for cancer detection (designated "PanCancer"), whereas *RARβ2*, *SCGB3A1*, *SEPT9* and *SOX17* were considered a gene panel for discrimination of primary cancer localization ("CancerType" panel). In ccfDNA, the "PanCancer" panel correctly detected 183 out of 253 cancer cases, corresponding to 72.4% sensitivity, 73.5% specificity and 72.8% accuracy (Table 13, Figure 15). Furthermore, "PanCancer" panel detected CRC stages 0, I and II with 78.38% sensitivity, 69.90% specificity, 48.33% PPV, 90.00% NPV and 72.14% accuracy, and early LC with 85.71% sensitivity, 75.73% specificity, 41.86 % PPV and 96.30% NPV.

Table 13. Biomarker performance detection of "PanCancer" panel (*APC, FOXA1* and *RASSF1A*) in ccfDNA.

	PanCancer
Sensitivity %	72.4
Specificity %	73.5
Positive Predictive Value %	87.1
Negative Predictive Value %	52.1
Accuracy %	72.8

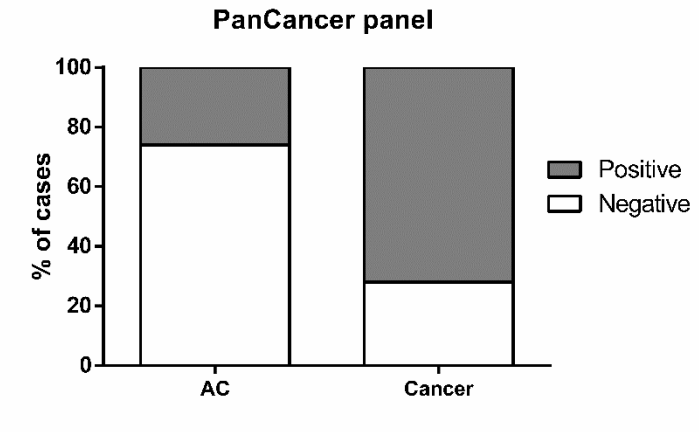

Figure 15. Percentage of cases identified by "PanCancer" panel in cancer samples (Positive 72%, Negative 28%) and ACs (Positive 26%, Negative 74%).

Using the "CancerType" panel, three methylated genes might be used to indicate the most likely primary location of the tumor detected by the "PanCancer" panel (Table 14, Table 15). *SCGB3A1* detected BrC with 80.69% specificity, whereas *SEPT9* methylation detected CRC with 98.90% specificity and *SOX17* detected LC with 85.56% specificity (Table 15). *RARβ2* was not further included in "CancerType" since it was not useful for discrimination between CRC and LC (data not shown). The results of the "CancerType" panel could, then, be used to select the best strategy for identification of primary localization (mammography, colonoscopy or LDCT).

Table 14. Methylated gene promoter combinations for BrC, CRC and LC discrimination using the "CancerType" panel.

Gene	BrC	CRC	LC
SCGB3A1	+	–	–
SEPT9	–	+	–
SOX17	–	–	+

"+" indicates a higher probability to find that cancer; "–" denotes that there is a low probability for that cancer type be present. Abbreviations: BrC – Breast Cancer; CRC – Colorectal Cancer; LC – Lung Cancer

Table 15. Performance of gene promoter combinations for discrimination among BrC, CRC and LC ("CancerType" panel).

		SCGB3A1	***SEPT9***	***SOX17***
Breast cancer	Sensitivity %	16.8	-	-
	Specificity %	80.0	-	-
	Accuracy %	53.0	-	-
Colorectal cancer	Sensitivity %	-	11.1	-
	Specificity %	-	98.9	-
	Accuracy %	-	73.9	-
Lung cancer	Sensitivity %	-	-	39.4
	Specificity %	-	-	85.1
	Accuracy %	-	-	71.9

DISCUSSION

BrC, CRC and LC are the most incident and lethal neoplasms among women in developed regions of the globe (1) and screening programs may decrease mortality through increased detection of early stage disease (17, 50, 176). Mammography and colonoscopy are gold-standard for BrC and CRC screening, whereas LDCT is recommended for high-risk smokers' screening (17, 52, 62). Notwithstanding, these screening tools have significant limitations, comprising risk of overdiagnosis/overtreatment, invasiveness and high cost, entailing low compliance, and suboptimal specificity, requiring further testing and increasing suspects' anxiety (19, 50). Hence, low-invasive screening strategies, capable of better triaging cancer suspects for testing with highly specific methods is an important clinical challenge. Owing to the ubiquity and cancer-specificity of selected aberrant gene promoter methylation, enabling successful cancer detection in liquid biopsies (95), we assessed the feasibility of ccfDNA analysis using multiplex qMSP for simultaneous BrC, CRC and LC detection in women.

Gene methylation levels were assessed in ccfDNA extracted from plasma of BrC, CRC and LC patients and ACs. The amount and quality of plasma ccfDNA is low, and the sodium-bisulfite modification leads to further DNA degradation, which hinders ccfDNA methylation analysis (171). In this study, due to sample collection limitations, ccfDNA was extracted from 3 mL of plasma, a reduced amount comparing with other studies (111, 177). Nonetheless, WGA of bisulfite-converted ccfDNA allowed to increase DNA quantity and, thus, multiplex qMSP sensitivity. Several reports showed that WGA produce unbiased amplified products that can be used in methylation analysis when the DNA quantity is limited (172, 178).

MethyLight qMSP is one of the most commonly used method for assessing genes' methylation status (112, 135). It requires primers and probes designed specifically for the bisulfite-converted DNA sequence. Although MethyLight is useful in methylation studies, it allows for single gene analysis, which can be limiting for samples with low DNA quantity. A multiplex qMSP reaction allows for the analysis of several targets in a single PCR reaction (179), hence it displays several advantages: (i) requires low amounts of DNA; (ii) is a faster and more efficient method than singleplex qMSP and (iii) less amounts of reagents are necessary (180), which is suitable to study DNA methylation in ccfDNA extracted from plasma/serum (155). Indeed, its usefulness in ccfDNA liquid biopsies was previously demonstrated (109, 113, 155). In this study we assessed the promoters' methylation levels of 9 genes plus *β-Actin* as a reference gene using a four-color multiplex assay. Nevertheless, primers and probes design might be one of the limitations associated with multiplex qMSP, since it is important to avoid non-specific links between primers and probes, and to assure target gene specificity. Furthermore, the ABI 7500 Real-Time PCR

system used in this study only allows five different fluorescent dyes (passive dye ROX plus four fluorescent dyes). Still, this limitation might be overcome by using platforms with more fluorescent channels (179). Importantly, herein, we demonstrate that multiplex qMSP is useful for analyzing in samples with minutes amounts of DNA, such as ccfDNA extracted from plasma/serum.

Candidate genes were selected based on an extensive and critical literature review, including our previously published results (166-168), and, globally, our findings are mostly in line with previous publications. For BrC, we confirmed *APC*, *FOXA1*, *RASSF1A* and *SCGB3A1* hypermethylation in ccfDNA, in accordance with published studies (111, 112, 130, 135), whereas *RARβ2* methylation findings paralleled some previous studies (111, 132, 181), but not others, either in tissue (180), fine-needle washings (167) or serum (127, 135, 182). Differences in methodology (127), population (135) and/or biological sample type (167, 180) likely explain these dissimilarities. Furthermore, *SOX17* promoter has been reported as aberrantly methylated in ccfDNA and CTCs from BrC patients' (124, 183), albeit its BrC biomarker potential requires further investigation. As for CRC, the significantly higher *APC*, *FOXA1*, *RARβ2*, *RASSF1A*, *SCGB3A1*, *SEPT9* and *SOX17* methylation levels in cancer patients are in line with previous publications (168, 184-189), although divergent results have been reported for *MGMT* (115, 168, 190). Concerning LC, and except for *SHOX2,* our results are in accordance with previous studies (114, 123, 126, 163, 191-193). To the best of our knowledge, this is the first study disclosing *FOXA1* methylation in CRC and LC patients' ccfDNA.

Some interesting clinicopathological correlates with gene promoter methylation status were disclosed. The association of *RASSF1A* promoter methylation with PR status parallels previous reports (194-196) and higher *RARβ2* methylation in node-positive BrC is in line with previous findings in sentinel lymph node metastasis (197, 198). Furthermore, some of the tested candidate genes might also convey relevant prognostic information, as *APC*, *SEPT9*, *SHOX2* and *SOX17* methylation levels were increased in CRC patients with distant metastasis. Interestingly, a recent study disclosed higher *SEPT9* and *SHOX2* methylation levels in ccfDNA of CRC patients with distant metastasis and advanced stages (199). Moreover, a correlation between *APC* methylation and more advanced CRC stage was previously established in CRC tissue analysis (200) and *APC* methylation was also found in CRC hepatic metastasis (201). Another interesting finding was the higher *APC* and *RARβ2* methylation levels in patients with SCLC *vs.* lung adenocarcinoma. Recently, a microRNA-based test (miRview®) test was approved for discrimination among LC subtypes (202), based on analysis of pre-operative biopsies, which might be difficult to obtain. Thus, gene promoter methylation assessment in ccfDNA might prove advantageous in lung

tumors with difficult access, since SCLC requires a specific treatment regimen and is associated with worse prognosis (203). Furthermore, an association between *RASSF1A* methylation and node-positive LC patients was found, which is in accordance with previous publications demonstrating higher *RASSF1A* methylation levels in more advanced tumor stage, associating with local recurrence and worse prognosis in LC patients (204, 205). Finally, *SOX17* promoter methylation levels associated with distant metastasis, in agreement with previous studies using plasma samples from LC patients (123).

Although several gene methylation panels have been proposed for specific cancer detection using ccfDNA (111-114), our main goal was to devise a gene panel enabling the simultaneous detection of the three most common cancers among women, thus potentially increasing the cost-effectiveness of a methylation-based screening test. Remarkably, similar sensitivity and specificity was disclosed by the "PanCancer" panel (*APC*, *FOXA1* and *RASSF1A*) compared to other gene methylation panels proposed for individual BrC, CRC and LC detection (111-114). Compared to mammography, "PanCancer" discloses lower sensitivity and specificity (14), but it may result advantageous for triaging women for mammographic screening, eventually decreasing cumulative radiation exposure and costs, while increasing women's compliance. It would be interesting to ascertain whether the molecular test might provide more accurate screening results than mammography in women with high breast density, for which mammography is mostly ineffective. Optimally performed colonoscopy detects CRC with 58-75% sensitivity, depending on the localization of the tumor (206), and allows for confirmatory tumor biopsy and polyp removal (52). Nevertheless, it is a costly, invasive approach that requires prior preparation and sedation (52), whereas FOBT tests are non-invasive but have limited sensitivity and specificity (52). The "PanCancer" panel disclosed similar detection performance to colonoscopy and superior to fecal occult blood tests, constituting a minimally-invasive test, amenable for screening. Finally, "PanCancer" panel clearly outperformed LDCT for LC detection and might be favorably used in a pre-screening context, to better identify high-risk suspects of harboring LC (Figure 16). Interestingly, the "PanCancer" panel detected stage I and II LC with a high sensitivity and specificity, and may, thus, constitute a novel option for LC early detection. Furthermore, it is likely that lesions difficult to diagnose by imaging techniques might be detectable using the "PanCancer" panel as *Shan et al.* have previously demonstrated that a methylation-based panel detected small breast tumors (< 1 cm) with higher sensitivity than mammography (112).

Identifying the putative cancer primary localization following a positive "PanCancer" panel result constitutes the next challenge. Based on the individual performance of the remainder gene promoters tested, we proposed another panel ("CancerType") which

attempts to indicate the most likely topography of the primary tumor. To increase cost-effectiveness, this panel would only be performed in “PanCancer” positive cases, allowing cancer suspects to be directed for mammography, colonoscopy or LDCT (Figure 16). Although “CancerType” genes individually display low sensitivity, the main goal of this panel is to discriminate among the three cancer types, requiring high specificity. Risk factors should be also considered (*e.g.*, familial history of BrC or CRC, tobacco exposure) to improve the detection strategy. In cases in which no tumor is found, looking for the remainder possible localizations should be guided according to clinical evaluation. Repeat testing after a defined time could also be considered. It is difficult, however, to estimate how results of the “CancerType” panel would perform in a real setting as it assessment implies a carefully designed study with relatively long follow-up period.

The main limitations of this study are the limited number of samples tested and the lack of long term follow-up, which would be required to determine whether asymptomatic controls testing positive would subsequently develop BrC, CRC or LC. These limitations also preclude an accurate estimate of the use of the two gene panels in a “real world” scenario. Nevertheless, it should be emphasized that our proposal is innovative and might foster the development of more accurate and cost-effective tools for BrC, CRC and LC screening.

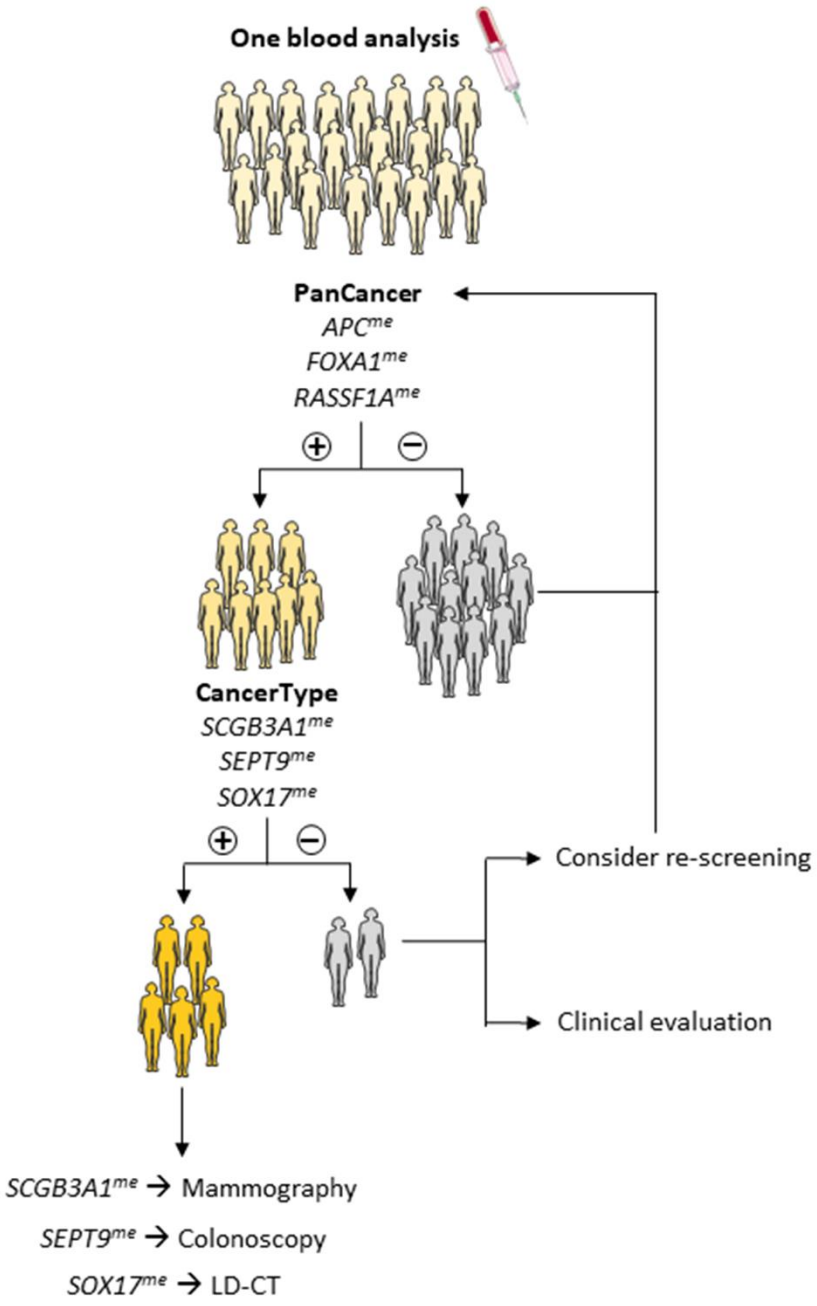

Figure 16. Schematic representation of a proposed algorithm for screening and management of breast, colorectal and lung cancers using the methylation panels. If “PanCancer” panel positive, “CancerType” panel would be performed in order to determine the cancer type present. After “CancerType”, exams such as mammography, colonoscopy or low-dose computed tomography (LDCT) would be executed to confirm the diagnosis. If “PanCancer” panel negative, a re-screening would be proposed, whereas if “CancerType” panel negative, a clinical evaluation or a re-screening would be options. Nunes SP *unpublished*

CONCLUSION

A selected gene promoter methylation assessment in ccfDNA is promising for simultaneous screening of BrC, CRC and LC, the major causes of cancer-related morbidity and mortality in women. The panels might complement current screening modalities, perfecting the triage of cancer suspects, increasing compliance and cost-effectiveness. Large-scale studies are now required to validate these findings and define the best algorithm for clinical application of these minimally-invasive methylation-based tests.

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APPENDIX

APPENDIX I

Breast Cancer TNM Staging

Table 1. TNM classification of BrC according to AJCC/UICC guidelines (Adapted from (27)).

T – Primary Tumor (Clinical and Pathological)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis (DCIS)	Ductal carcinoma *in situ*
T1	Tumor ≤ 20 mm in greatest dimension
T2	Tumor >20 and ≤ 50 mm in greatest dimension
T3	Tumor > 50 in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin; Inflammatory carcinoma
N – Regional Lymph Nodes (Clinical and Pathological)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional node metastases
N1	cN1* Metastasis to movable ipsilateral Level I, II axillary lymph node(s)
	pN1** Micrometastasis or macrometastasis in 1-3 axillary lymph nodes
N2	cN2 Metastasis in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted or ipsilateral internal mammary nodes in the absence of axillary lymph node metastasis
	pN2 Metastasis in 4-9 axillary lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in the absence of axillary node metastasis
N3	cN3 Metastasis in ipsilateral infraclavicular lymph node(s), ipsilateral internal mammary lymph node metastasis or metastasis in ipsilateral supraclavicular lymph node(s)
	pN3 Metastasis in 10 or more axillary lymph nodes or in infraclavicular lymph nodes, positive ipsilateral internal mammary lymph nodes by imaging in the presence of one or more positive Level I, II axillary nodes, more than 3 axillary lymph node metastasis by sentinel lymph node biopsy in clinically negative ipsilateral internal mammary lymph nodes or in ipsilateral supraclavicular lymph nodes
M – Distant Metastasis (Clinical and Pathological)	
M0	No clinical or radiographic evidence of distant metastasis
M1	Distant metastasis detected by clinical and radiographic means and/or histologically proven metastasis larger than 0.2mm

* Clinical stage; ** Pathological stage; DCIS – Ductal carcinoma *in situ*

Table 2. BrC staging with the correspondent TNM classification. Adapted from (27).

Primary Tumor (T)	Regional Lymph Node (N)	Distant Metastasis (M)	Stage
Tis	N0	M0	0
T1	N0	M0	IA
T0	N1mi	M0	IB
T1	N1mi	M0	IB
T0	N1	M0	IIA
T1	N1	M0	IIA
T2	N0	M0	IIA
T2	N1	M0	IIB
T3	N0	M0	IIB
T0	N2	M0	IIIA
T1	N2	M0	IIIA
T2	N2	M0	IIIA
T3	N1	M0	IIIA
T3	N2	M0	IIIA
T4	N0	M0	IIIB
T4	N1	M0	IIIB
T4	N2	M0	IIIB
Any T	N3	M0	IIIC
Any T	Any N	M1	IV

APPENDIX II

Colorectal Cancer TNM Staging

Table 3. TNM classification of CRC according to AJCC/UICC guidelines (Adapted from (27)).

T – Primary Tumor	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*, intramucosal carcinoma
T1	Tumor invades the submucosa (through the muscularis mucosa but not into muscularis propria)
T2	Tumor invades the muscularis propria
T3	Tumor invades through the muscularis propria into pericolorectal tissues
T4	Tumor invades the visceral peritoneum or invades or adheres to adjacent organ or structure
	T4a Tumor invades through the visceral peritoneum
	T4b Tumor directly invades or adheres to adjacent organs or structures
N – Regional Lymph Nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional node metastases
N1	One or three regional lymph nodes are positive, or any number of tumor deposits are present and all identifiable lymph nodes are negative
	N1a One regional lymph node is positive
	N1b Two or three regional nodes are positive
	N1c No regional nodes are positive, but there are tumor deposits in the subserosa, mesentery or nonperitonealized pericolic, or perirectal/mesorectal tissues
N2	Four or more regional nodes are positive
	N2a Four to six regional lymph nodes are positive
	N2b Seven or more regional lymph nodes are positive
M – Distant Metastasis	
M0	No distant metastasis by imaging
M1	Metastasis to one or more distant sides or organs or peritoneal metastasis is identified
	M1a Metastasis to one site or organ is identified without peritoneal metastasis
	M1b Metastasis to two or more sites or organs is identified without peritoneal metastasis
	M1c Metastasis to the peritoneal surface is identified alone or with other site or organ metastasis

Table 4. CRC staging with the correspondent TNM classification. Adapted from (27).

Primary Tumor (T)	Regional Lymph Node (N)	Distant Metastasis (M)	Stage
Tis	N0	M0	0
T1, T2	N0	M0	I
T3	N0	M0	IIA
T4a	N0	M0	IIB
T4b	N0	M0	IIC
T1, T2	N1/N1c	M0	IIIA
T1	N2a	M0	IIIA
T3-T4a	N1/N1c	M0	IIIB
T2-T3	N2a	M0	IIIB
T1-T2	N2b	M0	IIIB
T4a	N2a	M0	IIIC
T3-T4a	N2b	M0	IIIC
T4b	N1-N2	M0	IIIC
Any T	Any N	M1a	IVA
Any T	Any N	M1b	IVB
Any T	Any N	M1c	IVC

APPENDIX III

Lung Cancer TNM Staging

Table 5. TNM classification of LC according to AJCC/UICC guidelines (Adapted from [25]).

T – Primary Tumor	
Tx	Primary tumor cannot be assessed, or tumor proven by the presence of malignant cells in sputum or bronchial washing but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma *in situ* Squamous cell carcinoma *in situ* (SCIS) Adenocarcinoma *in situ* (AIS): adenocarcinoma with pure lepidic pattern. ≤3 cm in greatest dimension
T1	Tumor ≤3 in greatest dimension, surrounded by lung or visceral pleura without bronchoscopic evidence of invasion more proximal than lobar bronchus
	T1mi Minimally invasive adenocarcinoma: adenocarcinoma (≤3 cm in greatest dimension) with a predominantly lepidic pattern and ≤5 mm invasion in greatest dimension
	T1a Tumor ≤1 cm in greatest dimension. A superficial, spreading tumor of any size whose invasive component is limited to the bronchial wall and may extend proximal to the main bronchus also is classified as T1a, but these tumors are uncommon
	T1b Tumor >1cm but ≤2 cm in greatest dimension
	T1c Tumor >2 cm but ≤3 cm in greatest dimension
T2	Tumor >3 cm but ≤5 cm or having any of the following features: • Involves the main bronchus regardless of distance to the carina, but without involvement of the carina; • Invades visceral plaura; • Associated with atelectasis or obstructive pneumonitis that extends to the hiliar region, involving part or all of the lung.
	T2a Tumor >3 cm but ≤4 cm in greatest dimension
	T2b Tumor >4 cm but ≤5 cm in greatest dimension
T3	Tumor >5 cm but ≤7 cm in greatest dimension or directly invading any of the following: parietal pleura, chest wall, pherenic nerve, parietal pericardium; or separate tumor nodule(s) in the same lobe as the primary
T4	Tumor >7 cm or tumor of any size invading one or more of the following: diaphragm, mediastinum, heart, esophagus, vertebral body, or carina; separate tumor nodule(s) in an ipsilateral lobe different from that of the primary
N – Regional Lymph Nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional node metastases
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hiliar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral, hiliar, ipsilateral or contralateral hiliar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
M – Distant Metastasis	

M0	No distant metastasis
M1	Distant metastasis
	M1a Separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodules or malignant pleural or pericardial effusion
	M1b Single extrathoracic metastasis in a single organ (including involvement of a single nonregional node)
	M1c Multiple extrathoracic metastasis in a single organ or in multiple organs

Table 6. LC staging with the correspondent TNM classification. Adapted from [25].

Primary Tumor (T)	Regional Lymph Node (N)	Distant Metastasis (M)	Stage
TX	N0	M0	Occult carcinoma
Tis	N0	M0	0
T1mi	N0	M0	IA1
T1a	N0	M0	IA1
T1a	N1	M0	IIB
T1a	N2	M0	IIIA
T1a	N3	M0	IIIB
T1b	N0	M0	IA2
T1b	N1	M0	IIB
T1b	N2	M0	IIIA
T1b	N3	M0	IIIB
T1c	N0	M0	IA3
T1c	N1	M0	IIB
T1c	N2	M0	IIIA
T1c	N3	M0	IIIB
T2a	N0	M0	IB
T2a	N1	M0	IIB
T2a	N2	M0	IIIA
T2a	N3	M0	IIIB
T2b	N0	M0	IIA
T2b	N1	M0	IIB
T2b	N2	M0	IIIA
T2b	N3	M0	IIIB
T3	N0	M0	IIB
T3	N1	M0	IIIA
T3	N2	M0	IIIB

T3	N3	M0	IIIC
T4	N0	M0	IIIA
T4	N1	M0	IIIA
T4	N2	M0	IIIB
T4	N3	M0	IIIC
Any T	Any N	M1a	IVA
Any T	Any N	M1b	IVB
Any T	Any N	M1c	IVC

APPENDIX IV

Association between promoter's methylation levels and clinicopathological features in BrC ccfDNA

Table 7. Associations between BrC patients' clinicopathological features and *APC, FOXA1, MGMT, RARβ2, RASSF1A, SEPT9, SHOX2, SCGB3A1* and *SOX17* promoters' methylation levels.

Genes	*APC*	*FOXA1*	*MGMT*	*RARβ2*	*RASSF1A*	*SEPT9*	*SHOX2*	*SCGB3A1*	*SOX17*
Clinicopathological Features	*p* value								
Histological Type	0.483	0.423	0.949	0.409	0.351	0.357	0.725	0.279	0.141
Estrogen Receptor Status	0.341	0.697	0.685	0.975	0.090	0.564	0.726	0.478	0.675
Progesterone Receptor Status	0.826	0.358	0.586	0.590	**0.031**	0.439	0.769	0.513	0.285
Molecular Subtype	0.741	0.706	0.925	0.231	0.451	0.855	0.520	0.562	0.785
Primary Tumor (T)	0.398	0.926	0.773	0.445	0.134	0.138	0.567	0.529	0.275
Regional Node (N)	0.139	0.477	0.326	**0.008**	0.830	0.326	0.483	0.462	0.574
Clinical Stage	0.450	0.339	0.763	0.341	0.423	0.345	0.663	0.668	0.401

p values obtained by Mann-Whitney Test for Estrogen Receptor Status, Progesterone Receptor Status and Regional Node (N), and by Kruskal-Wallis Test for Histological Subtype, Molecular Subtype, Primary Tumor (T), and Clinical Stage. Distant Metastasis (M) was not evaluated due to the low number of M1 patients.

APPENDIX V

Association between promoter's methylation levels and clinicopathological features in CRC ccfDNA

Table 8. Associations between CRC patients' clinicopathological features and *APC*, *FOXA1*, *MGMT*, *RARβ2*, *RASSF1A*, *SEPT9*, *SHOX2*, *SCGB3A1* and *SOX17* promoters' methylation levels.

Genes	***APC***	***FOXA1***	***MGMT***	***RARβ2***	***RASSF1A***	***SEPT9***	***SHOX2***	***SCGB3A1***	***SOX17***
Clinicopathological Features	*p* value								
Tumor Location	0.808	0.499	0.325	0.192	0.499	0.201	0.120	0.501	0.054
Primary Tumor (T)	0.306	0.439	0.253	0.075	0.051	0.505	0.343	0.649	0.544
Regional Node (N)	0.601	0.270	0.382	0.179	0.828	0.720	0.355	0.418	0.908
Distant Metastasis (M)	**0.028**	0.569	0.191	0.863	0.826	**<0.0001**	**0.011**	0.733	**0.024**
Clinical Stage	0.202	0.963	0.462	0.901	0.387	**<0.0001**	0.131	0.585	0.152

p values obtained by Mann-Whitney Test for Regional Node (N) and Distant Metastasis (M), and by Kruskal-Wallis Test for Tumor Location, Primary Tumor (T) and Clinical Stage. Histological type was not evaluated due to the low sample number of premalignant lesions and neurodocrine carcinoma.

APPENDIX VI

Association between promoter's methylation levels and clinicopathological features in LC ccfDNA

Table 9. Associations between LC patients' clinicopathological features and *APC, FOXA1, MGMT, RARβ2, RASSF1A, SEPT9, SHOX2, SCGB3A1* and *SOX17* promoters' methylation levels.

Genes	***APC***	***FOXA1***	***MGMT***	***RARβ2***	***RASSF1A***	***SEPT9***	***SHOX2***	***SCGB3A1***	***SOX17***
Clinicopathological Features	*p* value								
Histological Type	**0.005**	0.724	1.00	**0.035**	0.369	1.00	0.292	0.166	0.229
Primary Tumor (T)	0.488	0.355	1.00	0.257	0.742	1.00	0.668	0.876	0.378
Regional Node (N)	0.141	0.267	1.00	0.500	**0.018**	1.00	0.210	0.837	0.185
Distant Metastasis (M)	0.321	0.200	1.00	0.243	0.082	1.00	0.633	0.297	**0.029**
Clinical Stage	0.405	0.174	1.00	0.354	0.271	1.00	0.845	0.771	0.142

p values obtained by Mann-Whitney Test for Histological Type, Regional Node (N) and Distant Metastasis (M), and by Kruskal-Wallis Test for Primary Tumor (T) and Clinical Stage. For Histological Type, only Adenocarcinoma and Small-cell lung cancer were consider, since others subtypes presented a low sample number for statistical analysis.